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THE JOURNAL
OF
APPLIED BACTERIOLOGY

EDITED FOR THE SOCIETY

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A STERILE AGAR MEDIUM DISPENSER

BY T. R. G. GRAY

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SUMMARY: A method is described for aseptically dispensing definite volumes of agar media of known pH, thus eliminating the use of tubes or McCartney bottles for storage.

IN MAKING plate counts of bacteria it is essential to use the same volume of medium for each plate. This is usually achieved by distributing measured volumes into tubes or McCartney bottles which are then sterilized and stored until required. The pH of many media is adjusted by adding acid or alkali to the buffered medium before sterilization. This has proved difficult with the sea water media used for enumerating marine bacteria, because buffer solutions disturb the ionic balance and cause precipitation of certain salts (Zobell, 1941). Experiments with sea water media of various kinds have shown that if no buffer is added the pH alters in an unpredictable fashion during sterilization or subsequent melting. The addition of a calculated quantity of sterile acid or alkali to each individual bottle of molten medium immediately before use is one way of overcoming this difficulty. This can be tedious and time consuming when large numbers of bottles are involved.

The use of a sterile agar medium dispenser removes these difficulties, since the pH of the medium can be adjusted in bulk immediately prior to its distribution. As the medium is dispensed directly into Petri dishes, the need for McCartney bottles or tubes is eliminated.

The addition of heat labile substances, such as certain antibiotics and glucose, to agar media which are to be dispensed in known quantities is also simplified by using this apparatus.

Construction

Basically the apparatus (Fig. 1) is a modification of a conventional dispenser used for distributing diluents. A reservoir of molten agar (A), fitted with a double entry screw cap (I) and an air filter (H), supplies the medium to a glass burette (J), calibrated to deliver 20 ml volumes. The apparatus is entirely enclosed in a cabinet maintained at 45°. The dispenser is operated by means of two screw clips (L and M) which pass through the walls of the cabinet. The burette is connected to a plugged bottle (O) screwed into the base of the cabinet. This device can be made very easily by cutting the base off a 250 g screwnecked chemical bottle. This is plugged with a cotton wool bung (N) through which the jet (P) passes. The jet is housed in a removable plug (G) which projects through the floor of the cabinet. The cabinet is raised off the bench to a height of 12 in. and the space below is bounded by three walls, thus making a convenient chamber which can be sterilized by spraying before use.

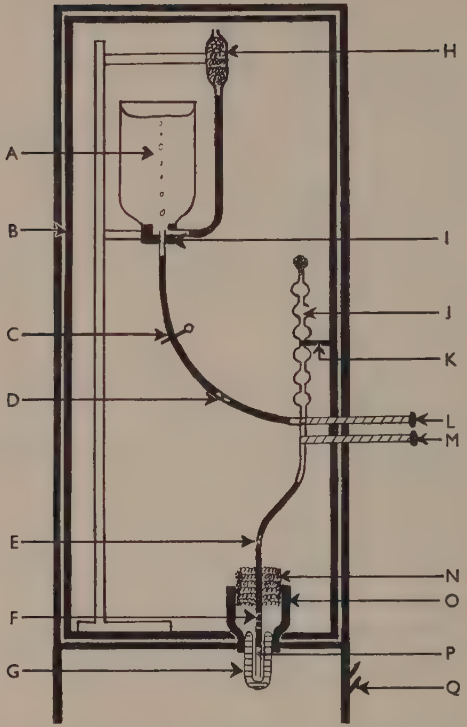

Fig. 1. Front view of the dispenser with the door removed. A, reservoir of molten agar; B, polystyrene foam block lagging; C, screw clip; D, E, F, adaptors; G, removable plug; H, air filter; I, double entry screw cap; J, glass burette; K, spring clip; L, M, screw clips; N, cotton wool bung; O, plugged bottle screwed into the base; P, jet; Q, switches on inoculating chamber wall.

The cabinet is maintained at 45° by four 200 W strip heaters in the floor and a 500 W asbestos covered heating wire threaded around the interior of the cabinet. These heaters are controlled by a Sunvic hot wire relay and thermostat (Sunvic Controls Ltd., London, W.C.2). The inside walls of the cabinet are made of aluminium and the outer walls of hardboard; between these is a lagging of polystyrene foam block (B). For convenience, an electric light was placed in the top of the cabinet; the light and the heaters are operated by means of switches at the base of the cabinet walls (Q).

Operation

The dispenser can be sterilized in three pieces normally connected by adaptors (D and E). The adaptors are protected by guard tubes during autoclaving. After autoclaving, the burette is fixed to the side wall of the cabinet by means of a spring clip (K) and the plugged bottle (O) is screwed into the base. The reservoir bottle, containing the molten medium which has been adjusted to the required pH, is clamped into a retort stand. The guard tubes are removed and the three component parts are joined. The screw clips (C, L and M) must be kept closed during this operation to prevent leakage of the medium.

To fill the burette, clips C and L are opened and when the burette has filled, L is closed. The plug (G) is then removed from the bottle and a Petri dish placed in position; clip M is then opened and 20 ml of agar allowed to pass into the dish. The whole operation may be viewed through a perspex panel in the door of the cabinet.

Should the jet (P) clog at any stage, due to solidification of the agar in the tube, it can be removed from the adaptor (F) with sterile forceps, and be replaced by another one.

After use, the reservoir should be changed for one of hot, sterile distilled water, and the apparatus washed with this water.

The author wishes to express grateful thanks to Professor C. G. C. Chesters and Mrs. M. Turner for their helpful comments, to Mr. D. Allen for his advice on the electrical circuits, and to Mr. H. Broadbent who constructed the cabinet. The work was carried out during the tenure of a Department of Scientific and Industrial Research Studentship.

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REFERENCE

ZOBELL, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *J. mar. Res.* **4**, 42.

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THE BACTERIOLOGY OF DISTANT WATER COD LANDED AT HULL

BY R. SPENCER

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SUMMARY: Plate counts at 20° on the skin and flesh of distant water cod landed at Hull were within the ranges $4{\cdot}2 \times 10^3$–$3{\cdot}6 \times 10^7$/cm^2 and $2{\cdot}3 \times 10^3$–$2{\cdot}4 \times 10^6$/g, respectively, with median values of approximately 10^6/cm^2 and 10^5/g. The counts at 37° were considerably lower. A light but general contamination of the fish with *Escherichia coli* I and the presence of occasional coagulase positive staphylococci were observed. Organisms of the salmonella-shigella groups were not detected. *Pseudomonas* and *Achromobacter* were the dominant genera and formed from 4% to 84%, and 3% to 65% of the total flora, respectively. Corynebacteria were usually present and in one case constituted 50% of the flesh flora. The significance of these findings in relation to proposed bacteriological standards for cleanliness of perishable foods and to the spoilage of fish is discussed.

THE OVERALL bacterial flora of freshly caught marine fish is by now well known. Earlier investigations have been reviewed by Shewan (1949) and more recent data have been given by Liston (1956, 1957) and Georgala (1957). Bacterial counts of fish surfaces range from 10^2 to 10^6/cm^2 with most values falling between 10^3 and 10^5/cm^2. The flesh is sterile but counts on the intestinal contents may reach 10^7/g. The preponderant bacterial genera are *Pseudomonas* and *Achromobacter*, with lower proportions of *Flavobacterium*, *Micrococcus*, and of coryneform bacteria. Members of the family Enterobacteriaceae and *Staphylococcus aureus* are normally absent. The majority of demersal fish (i.e. cod, haddock, plaice, etc.) consumed in Great Britain are not however 'freshly caught'. About two thirds are caught by trawlers in the distant waters of the Barents Sea, off Bear Island and Spitsbergen, and around Iceland. When caught, the fish are gutted and washed and stowed in ice for 4–18 days before reaching the ports. Considerable spoilage occurs during this period, with consequent changes in the bacterial flora. Handling on the trawler may also alter the flora of the fish. The results of laboratory experiments with a variety of fish have shown that the surface count may increase to 10^8–10^9/cm^2 during storage in ice. Furthermore, although it has been claimed that there is little bacterial invasion of the flesh until spoilage is well advanced (Dyer, Dyer & Snow, 1946), there is much evidence to show that the flesh may contain considerable numbers of bacteria in the relatively early stages (Shewan, 1949; Spencer, unpublished results). There are also qualitative changes in the flora during spoilage, the most recent data available indicating that the pseudomonads may increase at the expense of the achromobacters to form 90% of the flora after 10–12 days of storage (Shewan & Georgala, 1957), and there may be other modifications of the flora (Georgala, 1957), or the appearance of new types such as coli-aerogenes bacteria, including *Escherichia coli* (Spencer & Georgala, 1958), resulting from handling. As the fish landed at the ports is the raw material for the various processing operations carried out there, it is desirable to know what the flora of such fish is before considering bacteriological aspects of the various processes themselves. This was the aim of the present investigation.

The type of fish examined in the work reported here was cod, *Gadus callarias*, the species which constitutes almost 50% of the demersal fish landed in Great Britain. The fish were obtained from the Hull fish market where over 30% of the British demersal catch is landed (Ministry of Agriculture, Fisheries and Food, 1959).

MATERIALS AND METHODS

Fish. Because of commercial practices on the fish market it was not possible to obtain fish immediately the trawlers were unloaded. Consequently, fish were obtained from the premises of a large processor as soon as they arrived there from the market some 2–7 hr after unloading, which commences at 2.00 a.m. On unloading, the fish are placed in aluminium tubs, or kits, of 10 stones capacity, in which they are transported to the processors' premises. Any single fish selected for sampling was always the top one in a kit. Six fish were normally selected at a time and removed to the laboratory for sampling. A total of 130 fish from 20 vessels, and caught in all the major distant water fishing areas, were examined in detail. The period of the investigation extended from February, 1956 to January, 1958.

Sampling. A skin sample was taken from the untouched side of each fish by excising aseptically a circle of skin of area 4·9 cm^2, with as little adhering flesh as possible, from the shoulder of the fish above the lateral line. A cork borer was used as a template. The sample was placed in a screwcapped 2 oz bottle containing 10 ml of normal strength Ringer's solution plus a small amount of fine sand and was shaken mechanically for 10 min. This formed the first dilution.

To obtain the flesh sample, a complete fillet was removed from the fish and then skinned. Contamination of the fillet flesh was minimized by washing the fish before filleting to remove the surface slime, by careful cleaning of the filleting knife and the filleter's hands after each cut, and by preventing the flesh from coming into contact with the filleting board. The skinned fillet was collected in a sterile dish. In order to ensure a representative sample, three strips of flesh each approximately 1 in. wide were cut aseptically from the head, central and tail portions of the fillet and minced. A 50 g sample of this was then homogenized in 100 ml of normal strength Ringer's solution and 10 ml of the homogenate added to 90 ml of normal strength Ringer's solution to form the first dilution. Further decimal dilutions were made from this.

When examining for the presence of members of the family Enterobacteriaceae, swab samples were taken from fish in containers at the market, the visible surface (approximately one third of the total surface area) of the topmost fish in the container being swabbed.

Enumeration. Replicate plates were prepared from the serial dilutions of the skin and flesh samples and duplicates incubated at 37° and 20°, and also at 0° in some cases. For the 20° and 0° counts the medium consisted of: Lab-Lemco, 10 g;

peptone, 10 g; agar, 15 g; tap water, 250 ml; sea water, 750 ml; pH 7·6. For the 37° counts the medium was prepared with tap water only. Plates were incubated for 3 days at 37°, 5 days at 20° and 28 days at 0°, respectively.

The most probable numbers (MPN) of presumptive coli-aerogenes bacteria and *E. coli* I were estimated by the dilution method in MacConkey's broth (Oxoid) using the methods prescribed by the Ministry of Health (Report, 1956). After incubation for 48 hr at 37° to estimate the count of presumptive coli-aerogenes bacteria, all tubes showing a positive reaction were subcultured into fresh tubes of MacConkey's broth which were then incubated at 44° for 24 hr to estimate the count of presumptive *E. coli* I.

For the enumeration of coagulase positive *Staphylococcus aureus*, 0·1 ml volumes of the 10^{-1} and 10^{-2} dilutions of the skin samples were spread over the surface of mannitol-salt agar plates (Oxoid). After incubation at 37° for 48 hr the mannitol fermenting, orange pigmented, staphylococcus-like colonies were counted and all or a proportion of them picked for tube coagulase tests using rabbit plasma.

Swab samples taken from whole fish at the market were examined either for the presence of presumptive coli-aerogenes bacteria and *E. coli* I as above, with confirmation by IMViC tests of a proportion of the positive tubes, or for the presence of organisms of the salmonella-shigella groups by enrichment in selenite and tetrathionate broth followed, after 24 hr incubation, by subculture into deoxycholate and bismuth sulphite agars.

Classification of isolates. Isolations were made from the skin and flesh plates obtained at 20° from four batches of six fish. Isolations were also made from one set of plates obtained at 37° and 0°, respectively. Colonies were selected at random from the plates: normally ten colonies from each of the duplicate plates containing 50–500 colonies were selected, for each of the six samples in a batch. The cultures were inoculated into broth and subsequently plated out to test for purity and to determine the colonial characteristics. Pure cultures were examined morphologically by Gram's method and by phase contrast microscopy, for motility and for flagellation where necessary, and for sensitivity to a number of antibiotics (Shewan, Hodgkiss & Liston, 1954). The biochemical tests included liquefaction of gelatin, action in litmus milk, reduction of nitrate (Griess-Ilosvay method, Wilson & Miles, 1955) and reduction of trimethylamine oxide (Wood & Baird, 1943). With the exception of cultures from the plates obtained at 20° which were also tested for their ability to grow on nutrient agar at 0°, incubation was at 20°. The gelatin and litmus milk cultures were incubated for up to 28 days and the nitrate and trimethylamine oxide reduction tests were done after 7 and 9 days respectively.

RESULTS

Quantitative aspects. The distributions of the counts on the skin and flesh samples at 20° and 37° are shown in Figs. 1 and 2. Because of the wide range of counts obtained (for example, from less than 10^4 to greater than $10^7/\text{cm}^2$ for skin counts at 20°) it was not feasible to use equal intervals over the whole range. The intervals chosen, however, are considered to show the distributions satisfactorily and may also be of practical significance.

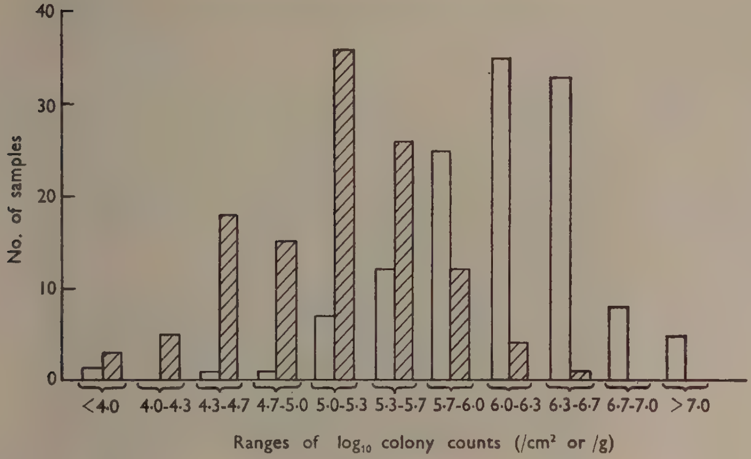

Fig. 1. The distribution of the colony counts obtained at 20° with skin (plain areas) and flesh (hatched areas) of commercial distant water cod.

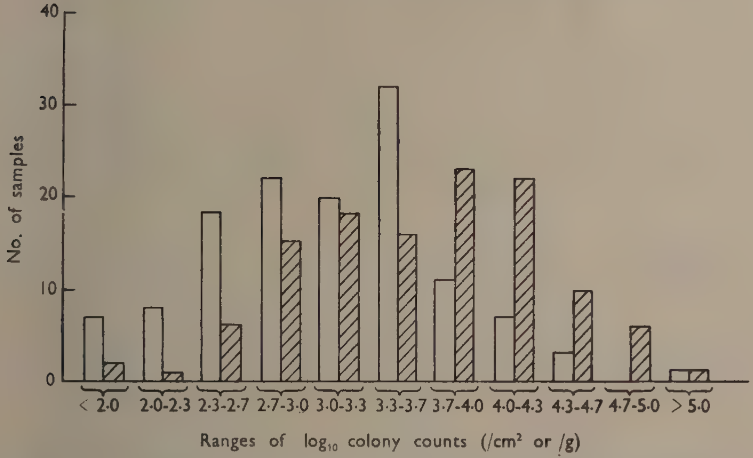

Fig. 2. The distribution of the colony counts obtained at 37° with skin (plain areas) and flesh (hatched areas) of commercial distant water cod.

It can be seen from Fig. 1 that more than 50% of the skin counts at 20° were greater than $10^6/\mathrm{cm}^2$ and that more than 50% of the flesh counts at 20° were greater than 10^5/g.

The counts at 37° (Fig. 2) were considerably lower than those at 20°, the usual result with such samples, but the ratios of counts at 37° to those at 20° were different for the skin and flesh samples, being about 1:1,000 and 1:10 to 1:100, respectively. Ratios of 1:100 to 1:1,000 are usual with marine materials, including fish stowed in ice, because of the preponderance of psychrophilic bacteria. The dissimilarity of the ratios for skin and flesh samples may be an indication of the presence of different types of bacteria in the two sets.

The occurrence of presumptive coli-aerogenes bacteria and *E. coli* I on skin and in flesh samples is shown in Tables 1 and 2. Presumptive coli-aerogenes bacteria were present on the skin in almost 95% of the fish examined, and *E. coli* I was present on two thirds of them, although the level of this organism seldom exceeded 2/cm². In the flesh samples the level of *E. coli* I did not exceed 3/g in approximately 95% of the samples.

Table 1. *The occurrence of presumptive coli-aerogenes organisms and* E. coli *I in swabs taken on several occasions from commercial distant water cod at the market*

	No. of fish examined	Samples (%) positive for	
		Coli-aerogenes bacteria	*E. coli* I
	103	100	92
	148	96	78
	150	99	68
	100	72	40
	120	100	87
	110	92	37
All fish	731	94	68

Table 2. *The distribution of ranges of numbers of presumptive coli-aerogenes bacteria and* E. coli *I in skin and flesh samples of commercial distant water cod*

Type of sample	Total samples	Ranges of MPN* (/cm² or /g)	Nos. (a) and percentages (b) in given ranges, of			
			Coli-aerogenes bacteria		*E. coli* I	
			(a)	(b)	(a)	(b)
Skin	96	<2	33	34	93	97
		2–10	39	41	3	3
		10–50	20	21		
		>50	4	4		
Flesh (Exp. 1)	30	<30	29	97	30	100
		>30	1	3		
Flesh (Exp. 2)	66	<3	52	79	62	95
		3–10	13	20	3	4
		>10	1	1	1	1

* MPN, most probable numbers.

Organisms of the salmonella-shigella groups were not detected during the examination of a total of 302 swabs taken on three occasions.

Eighty-six skin samples were examined for the presence of coagulase positive *Staphylococcus aureus*. During this examination 659 mannitol fermenting, yellow to orange pigmented colonies on mannitol-salt agar plates were examined and only 3 were coagulase positive.

Qualitative. More than 1,000 isolates from the plate counts at 0°, 20° and 37° on four batches of skin and flesh samples were examined in detail, the majority being from the plates obtained at 20°, and Table 3 shows the relative proportions of the various bacterial genera found.

In the single set of counts at 37° examined there was found the great preponderance of micrococci expected from previous work. The parallel counts at 0° and 20° on batch D revealed that the temperature of incubation of the plates caused only minor qualitative differences in the flora, namely fewer coryneforms and more achromobacters at 0° compared with 20°. This is in agreement with previous observations (Spencer, 1959).

Table 3. *The composition of the bacterial flora of the skin and flesh of commercial distant water cod*

Batch, fishing ground and date	Type of sample	Incub. temp.	No. of isolates examined	Percentage of					
				Pseudomonas	*Achromobacter*	*Flavobacterium*	*Corynebacterium*	*Micrococcus*	Miscellaneous*
A Bear Island February, 1956	Skin	20	116	16	52	14	15	0	3
		37	65	(——	19	——)	0	81	0
	Flesh	20	23	4	31	13	31	17	4
		37	42	(——	17	——)	0	83	0
B Norway Coast April, 1956	Skin	20	101	84	3	9	1	1	2
	Flesh	20	139	33	9	4	51	1	2
C Norway Coast March, 1957	Skin	20	115	57	26	0	15	2	0
	Flesh	20	109	43	39	0	16	2	0
D Bear Island June, 1957	Skin	20	106	58	30	2	10	0	0
		0	72	60	40	0	0	0	0
	Flesh	20	109	41	45	1	10	3	0
		0	60	28	65	7	0	0	0

* Yeasts and unclassified bacteria.

The skin flora at 20° showed the great preponderance of the genera *Pseudomonas* and *Achromobacter* previously found with fresh and spoiling fish. The proportions of these two genera, however, varied considerably from batch to batch, the former from 4% to 84% and the latter from 3% to 52% of the total flora. Although the flesh flora was almost identical to the skin flora with batch C (the minor differences being probably not significant), there were very marked differences with batches A and B where the proportions of corynebacteria in the flesh samples were considerably higher than in the skin samples. Corynebacteria were the predominant group in the flesh samples from batch B.

Table 4 shows the relative proportions within each genus, of isolates from the plate counts at 0° and 20° on the skin and flesh samples, which brought about certain biochemical reactions, and also includes the proportions of the 20° isolates which would grow at 0°. As expected, there were considerable variations in activity from genus to genus. The achromobacters, for example, seldom liquefied gelatin or peptonized litmus milk, whereas over half the pseudomonads and corynebacteria liquefied gelatin. Almost all the isolates from plates at 20° were capable of growing at 0°.

Table 4. *The proportions within each genus of organisms giving certain biochemical reactions, among isolates from plate counts at 20° and 0° on skin and flesh samples from commercial distant water cod*

Genus	No. of isolates	Percentage of isolates giving				
		Reduction of TMO*	Reduction of nitrate	Liquefaction of gelatin	Peptonization of litmus milk	Growth at 0°†
Pseudomonas	430	61	48	52	26	99
Achromobacter	303	36	90	13	6	97
Flavobacterium	37	38	65	92	73	100
Corynebacterium	157	53	73	54	11	90
Micrococcus	13	62	77	69	8	100
Miscellaneous‡	10	60	50	20	10	100
Total	950	51	67	41	19	97

* Trimethylamine oxide. † Only cultures isolated from plates incubated at 20° were tested. ‡ Yeasts and unclassified bacteria.

DISCUSSION

There can be no doubt that a large proportion of the demersal fish entering the processing and distribution chain in Britain, besides having surface counts at 20° of the order of 10^6 bacteria/cm^2, have flesh counts at 20° in excess of 10^5/g, a finding of considerable significance from the point of view of further processing and also when bacteriological standards for perishable foods are being considered.

A viable count standard of 250,000/g is being applied to filleted fish in Canada (Department of Fisheries of Canada, 1959) as a measure of factory hygiene, on the assumption that the flesh of the fish entering the factory is virtually sterile. It is obvious that such a standard could not be applied in this country as a considerable proportion of the fish entering the factory would have a flesh count in excess of that figure before processing. The use of fillets from such fish to produce frozen products such as fish sticks would result in counts at 20° in excess of 10^5/g in many cases.

Although few, if any, standards for total viable counts in foods have been promulgated, it has been suggested that perishable foods should not contain more than 10^5 bacteria/g because of the association of food poisoning with counts in excess of that figure (Buttiaux & Mossel, 1957). It is considered that such a standard is not applicable to unprocessed fish because the great majority of the bacteria present are psychrophilic and the multiplication which has caused the high count has normally taken place at temperatures well below those permitting the growth of food poisoning bacteria. This would also apply to such processed fish products as frozen fish and fish sticks, although possibly not to smoked fish and certainly not to fish cakes where the initial heat treatment sterilizes the fish and subsequent contamination would possibly not be by psychrophilic bacteria of marine origin.

The origin of the light, but general, contamination of the fish with coli-aerogenes bacteria and faecal coli is not known, but it is unlikely to be the ice going into the trawlers (Spencer, unpublished results), which is normally of a high standard of bacteriological purity. The possibility of direct contamination of the fishroom fittings, the ice, or the fish by personnel working in the fishroom cannot, however, be excluded, although it should be noted that multiplication of these organisms in the fish hold is unlikely because of the temperatures usually maintained there.

The information obtained on the proportions of the various bacterial types present on and in the fish do little to clarify the position regarding the importance of any one type in fish spoilage. It is clear that members of the genera *Pseudomonas* and *Achromobacter* were numerically the most important and, of the two, the pseudomonads were generally present in the larger numbers; these results are in accordance with those obtained in other circumstances. It was also confirmed that the pseudomonads associated with spoiling fish were biochemically more active than the achromobacters under the conditions and with the tests used, and it is reasonable to ascribe to this group of bacteria an important part in the spoilage of fish. In certain flesh samples, however, achromobacters and/or corynebacteria were present in larger numbers than pseudomonads, and as the corynebacteria were at least as biochemically active as the pseudomonads these groups may well play a part in spoilage in certain circumstances.

The work described in this paper was carried out as part of the programme of the Department of Scientific and Industrial Research. Grateful acknowledgements are due to Dr. J. H. McCoy, Public Health Laboratory Service, Hull, who examined swabs for the presence of organisms of the salmonella-shigella groups.

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DEVELOPMENTS IN APPARATUS FOR THE CULTURE OF MICRO-ORGANISMS: DESIGN FEATURES OF A LABORATORY TWO STAGE CONTINUOUS FLOW CULTURE APPARATUS

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SUMMARY: A two stage continuous flow apparatus with a first stage working volume of 2–3 l. and a second stage working volume of 6–14 l. is described.

Each stage has separate controls over the environmental factors; temperature, pH value and nutrient supply; and over foaming.

In the first stage the dilution rate is altered by changing the flow rate; in the second stage the dilution rate is altered by changing the working volume.

The apparatus has proved suitable for the continuous culture of a filamentous mould, which is more exacting from the engineering standpoint than culture of bacteria.

The publications by Monod (1950) and by Novick & Szilard (1950) marked a turning point in the history of continuous flow culture of micro-organisms. Not only did they describe practical apparatus, the Bactogen and the Chemostat, but they laid the whole technique on a firm theoretical basis which had previously been lacking. Before that time attempts at continuous culture had been crude and of limited application. After it, interest gradually increased as the general principles were applied to a variety of problems. The literature on the subject has been fully covered in the review by Řičica (1958).

Novick (1955), writing of continuous culture methods, has said: "Obviously more widespread application must await more widespread understanding of the principles and increased familiarity with the techniques. On the other hand, more widespread usage demands that the apparatus itself be simple in its construction and reliable in its operation". These sentences well express the aim of the present paper, which gives details of the design and technique of operation of a two stage continuous flow apparatus suitable for the culture of bacteria, yeasts, filamentous fungi and tissue cells on a laboratory scale.

During studies of the formation of exocellular products by a bacterium (Pirt & Callow, 1958*a*) it became clear that a single stage process, while permitting maximum conversion of substrate to cells, was inadequate for conversion of substrate to an extracellular product. This led to the design of a two stage apparatus in which optimum conditions for growth could be provided in the first stage and optimum conditions for product formation in the second. Some results with such an apparatus have already been published (Pirt & Callow, 1959*a*). Meanwhile our interest turned to the growth of filamentous moulds in continuous culture (Pirt & Callow, 1959*b*, 1960). A submerged culture of a filamentous fungus differs in behaviour from most bacterial cultures in several ways: it sediments rapidly unless continuously agitated; it has a marked effect on the apparent viscosity of the medium; and it has a much lower growth rate. These properties called for modifications to the culture apparatus which have been incorporated in the present design.

The idea of multistage fermentations is not a new one. Maxon (1955) gave a number of references to multivessel systems, most of which were concerned with the industrial production of alcohol. Recently, Beran (1958) described a two stage process for the production of baker's yeast from molasses using laboratory scale apparatus briefly described by Řičica (1958). Andreev (1958) gave some results obtained in a laboratory apparatus using two vessels in series with recycling of yeast, for alcohol production, and Dyr, Protiva & Praus (1958) investigated the acetone-butanol fermentation on a laboratory scale using up to five vessels in series. In spite of the obvious interest in multistage processes, no detailed description of the design and working of the necessary apparatus seems to have been given previously.

APPARATUS

A flow diagram of our two stage continuous culture apparatus is given in Fig. 1, and photographs in Plates 1 and 2. The apparatus consists essentially of two culture vessels in series, each vessel having independent control of aeration, temperature, pH and foaming, and its own substrate feed. The design of the vessels and some of the auxiliary equipment is based on previously published work (Elsworth, Meakin, Pirt & Capell, 1956; Elsworth, Capell & Telling, 1958). The pH control and foam control equipment is also as previously described (Callow & Pirt, 1956; Pirt & Callow, 1958*b*). Only such modifications as have been found necessary for a two stage apparatus or for fungal culture will be described in detail below.

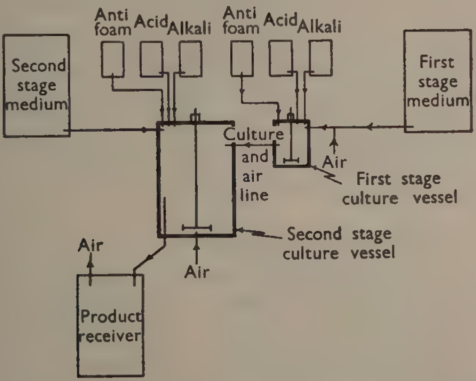

Fig. 1. Flow diagram of a two stage continuous culture system.

Size of culture vessels

In most two stage processes it is probable that a longer residence time in the second stage than in the first is desirable. The main reason for this is that formation of a high concentration of product often requires a longer time than growth of the organism. We therefore selected culture vessels with a first to second stage volume ratio of about 1:5. It should be noted, however, that for some applications, e.g. virus production, a reversal of these ratios may be necessary. For production on a larger scale it might be convenient to use a number of vessels in parallel for the second stage.

Several factors determine the absolute size of vessels selected. Economy of space and medium supply suggest a very small growth stage. On the other hand there is a minimum, practical size of vessel to which all the necessary control equipment can be fitted and it is also important that the total culture volume should not be so small that periodic sampling would cause a serious disturbance of the steady state. These considerations led us to construct first an apparatus having a vessel of 1 l. total capacity (diameter, 3·5 in.) for the first stage, and a vessel of 5 l. total capacity (diameter, 6 in.) (Elsworth *et al.* 1956) for the second stage. This apparatus (see Pirt & Callow, 1959*a*) was completely satisfactory for bacterial cultures.

Attempts to use the same apparatus with *Penicillium chrysogenum*, however, were less successful. The overflow tubes became clogged with mycelium which then acted as a filter allowing liquid to pass but not mycelium. Because of the narrowness of the 1 l. vessel, and consequent close proximity of the instruments installed in it, massive bridges of mycelium formed between the instruments and progressively slowed up movement of the culture. This did not occur in the 5 l. vessel. We therefore decided to use the original second stage vessel, which permitted adequate mixing and satisfactory growth of mycelium up to at least 35 mg/ml dry wt., for the growth stage and install a correspondingly larger vessel for the second stage. The new second stage vessel consisted of an 18 in. length of 9 in. diameter industrial glass pipeline with metal head and baseplates and had a total volume of about 18·5 l. Our experience indicates that 6 in. is the minimum practicable diameter for a vessel for the continuous culture of a filamentous mould.

Control of culture volume in the vessels

In any continuous culture apparatus an important variable is the dilution rate, i.e. the ratio of flow rate to culture volume, since this fixes the mean residence time and, at least in the first stage, controls the growth rate (see Herbert, 1958). The dilution rate in the first stage may be varied either by working at a fixed flow rate and varying the culture volume or by working at a fixed volume and varying the flow rate of medium. The latter is generally the more practical. In the second stage, however, no choice is available, since once the flow rate for the first stage has been fixed, only by varying the volume of the second stage can *its* dilution rate be varied. The simple, adjustable overflow tube described by Elsworth *et al.* (1956), which functioned well on the second stage vessel when growing bacteria, proved unsuitable when growing filamentous moulds. An adjustable internal weir (Fig. 2) was eventually constructed which has been thoroughly tested only for mould culture but which should prove equally suitable for any micro-organism. The weir consists of a length of stainless steel tubing, $\frac{5}{8}$ in. external diameter, which passes through a double O ring seal in the reactor baseplate. This allows the height of the weir to be adjusted from below. The exterior of the tube, of which a part may be either inside or outside the reactor, according to the desired volume, is protected from contact with the atmosphere by a rubber bellows fastened at one end to the reactor base and at the other end to the weir tube near its lower extremity. By this arrangement the culture volume in the second stage may be varied over the range 6–14 l.

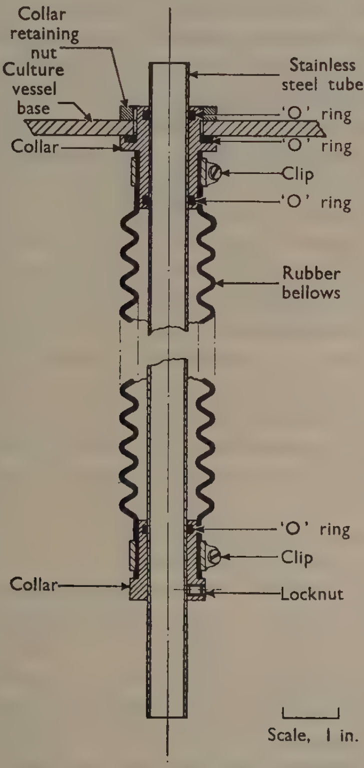
Collar retaining nut
Culture vessel base
Collar
Stainless steel tube
'O' ring
'O' ring
Clip
'O' ring
Rubber bellows
'O' ring
Clip
Collar
Locknut
Scale, 1 in.

Fig. 2. Adjustable internal weir for culture vessel.

The first stage has two take-off tubes sealed directly into the vessel wall. These are connected to the head plate of the second stage vessel by wide bore (16 mm) rubber tubing. The two levels permit a working volume of either 1·8 or 3·5 l. to be used. Each tube for the transfer of culture from the first stage to the second stage vessel was made as short as possible (25 cm) to make the transfer as fast as possible. There is a tendency for mycelium to accumulate and cause a blockage in the horizontal part of a take-off tube inserted in the vessel wall and a vertical tube inserted through the base is a preferable form of weir. The culture flows by gravity from the first stage to the second and from the second stage to the product receiver below.

Metering pumps and continuous liquid feed devices

The chemostat type of continuous culture apparatus requires a constant supply of medium fed at an accurately controlled but easily variable rate. A simple way of obtaining this is to use some variation of the Marriotte bottle such as that described by Elsworth *et al.* (1956). One defect of such an arrangement is that any variation in air pressure within the reactor will lead to variation in medium flow. Even a partial blockage of the air exit from the reactor may result in a complete stoppage of the flow of medium. With mould cultures it was found that, even with wide bore exits from the reactors, temporary blockages sometimes occurred. We have therefore used metering pumps, of which several suitable types are commercially available.

The present apparatus uses three types: a finger pump (Sigmamotor model T6S, Sigmamotor Inc., Middleport, N.Y.), a piston pump (DCL Micropump Mk 3, max. capacity 7 cm^3/hr; Distillers Co. Ltd., Great Burgh, Epsom, Surrey) and a motor-operated metering valve (Pirt & Callow, 1958*b*). In the first type a number of metal fingers descend consecutively onto a rubber tube and thus force liquid along. The flow rate is governed by the bore of the tubing and by the speed of operation of the fingers. Thus the same pumping unit is capable of giving a range of flow rates from a few millilitres to many litres/hr, particularly if fitted with some form of stepless, variable speed drive. Silicone rubber tubing with walls not less than 1·5 mm thick works well in the pump for aqueous solutions. Capillary silicone tubing of 0·5 mm bore (Esco (Rubber) Ltd., Somerford Grove, London, N.16) may be used for very low flow rates. By selecting a tube diameter which will give the desired flow rate at a low pump speed the tube wear is minimized. We usually work in the range 1–10 rev/min; this results in negligible wear.

The piston pump consists of a motor driven plunger in a cylinder fitted with double ball valves on the inlet and outlet. As normally supplied it operates at constant speed, the flow rate being controlled by the length of travel of the piston which is adjusted by micrometer. The disadvantages of this type of pump are: its rather narrow working range, the possibility of ball valve failure unless carefully filtered solutions are used, and the possibility of contamination through the piston gland. However, the pump has been used successfully to feed sodium phenylacetate solution into the penicillin fermentation. The only disadvantage of the finger pump is its greater size, particularly when fitted with variable speed drive. From the point of view of sterile operation over long periods it is definitely to be preferred, since there can be no possible contact between the interior and exterior of the system so long as the rubber tubing remains intact. It is also little affected by suspended solids in the medium.

By introducing into the medium supply line between the pump and the culture vessel an air stream (0·8 l./min) which expelled the medium, the growth of *Penicillium* cultures in the medium line was effectively prevented without use of the heat seal advocated by Elsworth *et al.* (1956).

Flow rate measurement

An important accessory to whatever system of continuous feed is used is the means of measuring the flow rate. For rates above about 30 ml/hr a vertical graduated tube (Elsworth *et al.* 1956) is simple and sufficiently accurate. For lower rates this method becomes tedious and involves stopping the supply to the culture for such a time as may be harmful. We have therefore adopted a method which makes use of a horizontal calibrated tube. This tube, which may conveniently be a 1 or 2 ml pipette, is inserted directly in the liquid inlet line between pump and reactor. A side tube sealed to the pipette at the pump end carries a small air filter and is normally clipped off. In order to make a flow check a small air bubble is blown into the liquid stream from the side tube. Coating the internal wall of the pipette with a silicone film ensures that the bubble travels freely without sticking. The flow rate can then be calculated from the rate of travel of the leading edge of the bubble along the calibrated tube. Here the interruption of flow caused by making the flow measurement is insignificant since it is only that caused by the bubble itself. This method has been used with rates down to 0·3 ml/hr.

A characteristic of finger pumps is that there is a slight suckback at one point in the cycle of operation of the fingers. This can lead to errors in calculating flow rates if the time for a fixed volume of liquid is used as a basis. For accurate results it is necessary to take the time over one complete cycle, or a number of cycles. After a short initial 'bedding in' of the fingers to a new piece of tubing the volume per cycle remains extremely constant so that, once this volume has been accurately measured, a revolution counter on the pump shaft may be used to check the flow rate.

A long term check on all liquids going into the reactors is obtained by having all feed reservoirs calibrated and recording the levels at sampling times.

Aeration and agitation

In any continuous culture apparatus, agitation is required for efficient mixing and also for gas transfer between the liquid and gaseous phases. Furthermore, in all common aeration systems the aeration system and the agitation system must be considered as one. When growing bacteria in the two stage apparatus, we used a conventional baffled system in both reactors. Agitation was provided by an eight blade, vaned disc impeller. Because the first stage is concerned with growth, very high oxygen solution rates may be necessary if oxygen is not to become a limiting factor. In the second stage, however, the oxygen demand will usually be much less. For the reasons given below it was found better to use vortex aeration (Chain *et al.* 1952). Sulphite oxidation values with vortex aeration are given in Figs. 3, 4 and 5. It should be noted that the oxygen solution rate was markedly dependent on the volume of solution and, with low volumes, the oxygen solution rates obtained are equal to the highest reported for the baffled system by Elsworth *et al.* (1956).

For fermentations with filamentous fungi the vortex system of aeration was used because the elimination of baffles considerably reduced the growth of mycelium on the reactor walls. This growth occurs to some extent even in batch runs but is particularly troublesome in continuous cultures of long duration. A further reduction in growth on the walls was obtained by running the reactor nearly full, thus reducing the area of wall above the culture fluid. This was tested in a single stage apparatus using a pipeline section 6 in. in diameter and 6 in. high for the reactor, with the agitated liquid level only 1 in. below the headplate. No difficulties were experienced by running the reactor in this way; the estimated reduction in growth on the walls was 75%. The short form of culture vessel has not yet been tried for the growth stage of a two stage apparatus. Since little growth normally takes place in the second stage, growth on the walls was less of a problem.

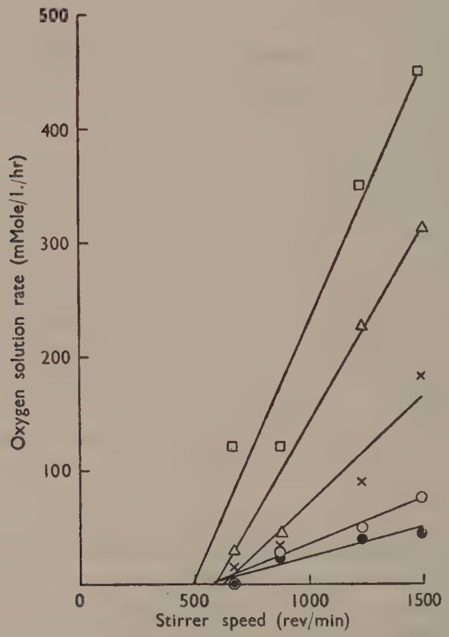

Fig. 3. Oxygen solution rates (Cu-catalyzed sulphite oxidation) in a 6 in. diameter vessel with vortex aeration and with an excess of air passed over the liquid surface. Impeller diameter, 63 mm; blade depth, 16 mm, 10 mm above the disc; height of the disc above the base of the culture vessel, 40 mm. Volumes (l.) of solution used: squares, 1·0; triangles, 1·5; crosses, 2·0; open circles, 2·5; full circles, 3·0.

The agitator drive assembly and the shaft seal were as described by Elsworth *et al* (1956). A similar system was used on both vessels. The longer shaft of the seconc stage was found to whip and vibrate, thus causing severe wear in the ferrobestos bearing. A footstep bearing to support the bottom of the shaft was therefore fittec to the internal face of the baseplate. A noncorrodible ballrace in a stainless stee housing was used and the only lubrication was that provided by the culture fluid in which the bearing was completely immersed. The usual running speed was 1,000 rev/min. The ballrace was replaced after it had run for 4,000 hr.

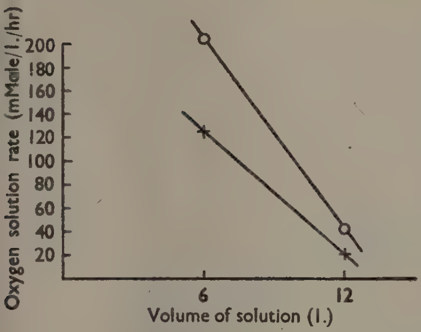

Fig. 4. The relationship between oxygen solution rate (Cu-catalyzed sulphite oxidation) and volume of solution in a 9 in. diameter vessel, with vortex aeration and with an excess of air passed over the surface. Impeller speed, 1,000 rev/min. Crosses: impeller diameter, 89 mm; blade depth, 30 mm, 20 mm above the disc; height of the disc above the base of the culture vessel, 65 mm. Open circles: impeller diameter, 102 mm; blade depth, 30 mm, 20 mm above the disc; height of the disc above the base of the culture vessel, 65 mm.

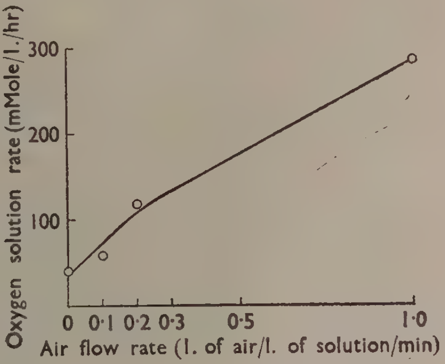

Fig. 5. The relationship between oxygen solution rate (Cu-catalyzed sulphite oxidation) and air flow rate in a 9 in. diameter vessel with vortex aeration. Volume of solution, 12 l.; impeller speed, 1,000 rev/min. Air was passed through the liquid from a single hole (3 mm diameter) sparger, 55 mm below the impeller disc. Impeller diameter, 102 mm; blade depth, 30 mm, 20 mm above the disc; height of the disc above the base of the vessel, 65 mm.

Control of pH

The system used for the automatic control of pH in the culture was that described previously (Callow & Pirt, 1956) with a few slight modifications. The quietness and reliability of the control valves were improved by fitting D.C. operated solenoids (Series L, model 1, rating 1; Westool Ltd., St. Helens, Auckland, Co. Durham), which may be used without the modification required by the A.C. solenoids. The alkali strength was increased to 4N to reduce the volume added. Overshooting of the control point when using the concentrated alkali was prevented by inserting in the solenoid circuit a time-pulse device (Wright, 1960). An air balance tube, to maintain the same overpressure above the acid and alkali as above the culture, was added. This was necessary in order that a temporary build up of pressure in the reactor should not counteract the small head of pressure from the acid and alkali reservoirs and thus stop the flow or force culture back up the feed lines.

Experience has shown that the estimate of several hundred hours originally given for the life of a glass electrode under continuous culture conditions was overcautious. In fact, all electrodes used by us in continuous culture in the last three years have lasted for more than 3,000 hr in continuous operation, that is about 4 months. In any case, a faulty electrode may be replaced without interrupting the run.

OPERATING TECHNIQUE

Sterilization

A continuous culture is normally expected to last for some weeks or even months The first step, therefore, in setting up the apparatus is to ensure as far as possibl its reliability. Between runs a thorough overhaul is made of all instruments mechanical components, shaft seals, tubing and gaskets. The apparatus is the prepared for sterilization in an autoclave, for which it is broken down into convenient sized units. The reservoirs and reactors are all sterilized empty. Afte sterilization the components are reassembled aseptically on the frame and all contro equipment connected.

When the assembly is complete, the medium reservoir is filled from a chargin bottle. The reactors are then filled to the desired working level from the reservoir using overpressure and bypassing the pump for rapid filling, and a small quantit of medium is allowed to flow over into the product receivers. The apparatus is no left for a suitable time, under working conditions, to check sterility. This perio also serves to permit any adjustments to be made to temperature and pH contro equipment if necessary.

Inoculation

Meanwhile a seed culture is prepared using conical flasks on a shaker. Sufficien inoculum may be prepared either for inoculation of both reactors simultaneousl or of the first stage only. At the appropriate time the inoculum is transferred fro the flask to the reactor through the inoculation port on the reactor head and th culture is grown batchwise. If only the first stage is to be inoculated from a flas the reactor is filled well above the normal working volume and the two stages kep isolated from each other until the batch growth is nearing completion in the firs stage. Communication is then established between the two stages so that cultur flows from the first stage to reduce it to its normal working volume and to inoculat the second stage. While the batch growth proceeds in the second stage the first stag is fed from the medium reservoir.

Samples of culture fluid may be withdrawn for analytical purposes through th sampling ports. A very convenient clip for use on wide bore rubber tubing is tha described by Chain *et al.* (1954).

Gas analyses

Gas analyses are routinely made with an Orsat apparatus on samples of effluent ga from each reactor. Air from the compressed air line at one atmosphere pressure i fed to the reactors through a reducing valve set to 3 lb/in^2. From the reducing valv each reactor is supplied through a separate needle valve, flowmeter (Rotamete Manufacturing Co., Croydon) and air filter. The combined effluent gas is also metered For analytical purposes the composition of the effluent from the second stage is firs found. The first stage is then isolated from the second so that the composition o the gas flowing into the second stage from the first may be determined. From thes measurements the oxygen consumption and the carbon dioxide output of each stag are calculated.

DISCUSSION

We have found that the culture vessel described by Elsworth *et al.* (1956) can be easily adapted for the culture of bacteria, filamentous moulds, protozoa, bacterial viruses, and animal tissue cells. The proven adaptability and the more extensive performance data make the stirred culture vessel generally preferable to other types of culture vessel such as the rotated vessel or the packed column.

The apparatus is capable of further development. As we have indicated, there are many problems in the design of a multistage continuous flow apparatus, and alternative solutions to them might be found.

The 9 in. diameter vessel was designed in collaboration with Mr. R. Elsworth and Mr. J. F. H. Peel. Mr. F. T. Stockwell collaborated in the design of the adjustable internal weir. Mr. J. E. D. Stratton has given excellent technical assistance. The workshops' staff contributed much to the success of the work. Dr. R. Falini, Istituto Superiore di Sanità, Rome, kindly supplied us with the sampling tube clamps.

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Explanation of Plates

Plate 1. Two stage continuous flow culture apparatus: the general lay out. A, instrument panel; B, air and water control panel; C, reservoir for medium; D, first stage culture vessel; E, second stage culture vessel; F, pump for medium; G, antifoam meter; H, antifoam pump; J, acid and alkali reservoirs; K, solenoid valves on acid and alkali lines; L, time-pulse device of acid and alkali solenoids; M, product receiver.

Plate 2. Detail showing the two culture vessels.

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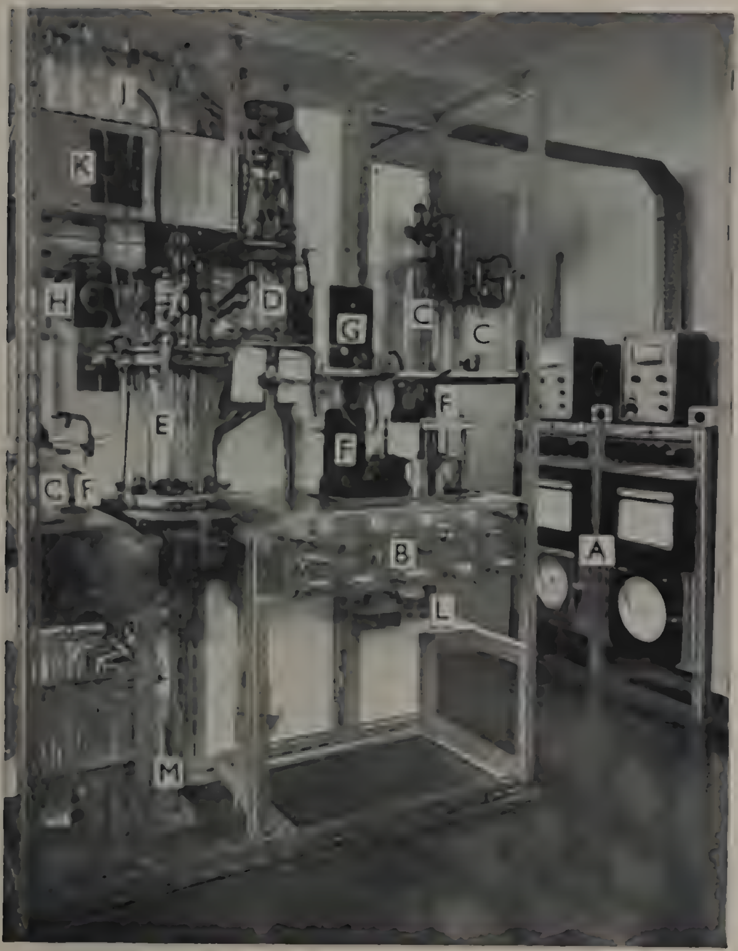
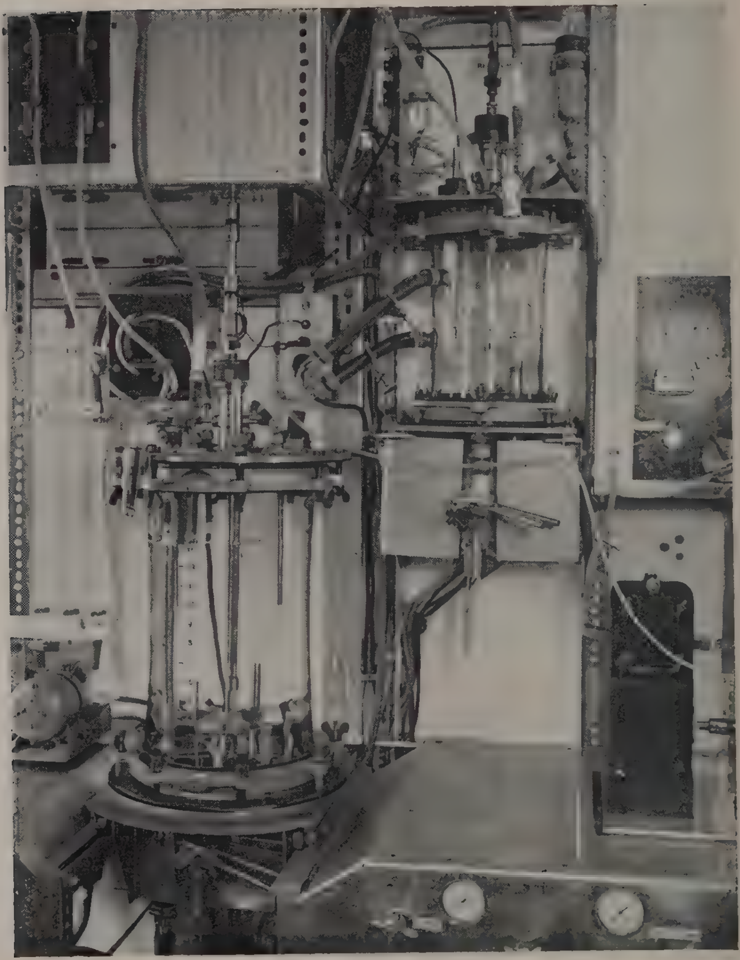


D. S. Callow & S. J. Pirt—Developments in Apparatus for the Culture of Micro-organisms: Design Features of a Laboratory Two Stage Continuous Flow Culture Apparatus. Plate 1



D. S. Callow & S. J. Pirt—Developments in Apparatus for the Culture of Micro-organisms: Design Features of a Laboratory Two Stage Continuous Flow Culture Apparatus. Plate 2

PARK, R. W. A. (1961). *J. appl. Bact.* **24** (1), 23–26.

A NOTE ON THE SYSTEMATIC POSITION OF *VIBRIO FETUS*

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SUMMARY: The morphology of *Vibrio fetus*, and of some nonpathogenic vibrios isolated from cattle, has been compared with that of 5 type cultures of *Vibrio* spp., including *V. cholerae*, and the systematic position of these bovine vibrios reviewed. Morphological differences between *V. fetus* and the nonpathogenic bovine vibrios have been observed. These may be of assistance in their identification.

THE ORGANISM known as *Vibrio fetus*, which causes abortion and sterility in cattle and abortion in sheep, was originally placed in the genus *Vibrio* by Smith & Taylor (1919) on the grounds that young cultures consisted of comma shaped bacteria having single polar flagella. This classification, however, appears to be questionable in view of the microaerophilic nature of *V. fetus* (Smith, 1918; Kiggins & Plastridge, 1956) and of the obligate requirement which it displays for a higher concentration of CO_2 than is present in the atmosphere (Kiggins & Plastridge, 1956). As it has been shown that curved rods do not necessarily belong to the genus *Vibrio* (Laughton, 1948; Park & Jeynes, 1959) and that most rod shaped bacteria display a certain degree of curvature (Pijper, 1946), it was thought that a reassessment of the systematic position of *V. fetus* would be of interest. In addition, it was hoped that differences between the morphology of *V. fetus* and the nonpathogenic bovine vibrios might be revealed and find practical application in the diagnosis of disease in cattle.

METHODS

Cultures examined. The following organisms were obtained from the National Collection of Type Cultures: NCTC 4693 (*V. cholerae*); and NCTC 8042, 4711, 4715 and 4716, representing respectively the *Vibrio* groups 2, 3, 5 and 6 of Gardner & Venkatraman, 1935). These will be referred to as the aerobic vibrios. Three strains of *V. fetus*, F5/1, EXT-1 and EXT-2, and 3 strains of nonpathogenic vibrios isolated from cattle, CMC3, DT and BSM3, were kindly supplied by Dr. W. J. B. Morgan. These will be referred to as the bovine vibrios.

Cultivation of organisms and media used. Monthly subcultures of the aerobic vibrios were made on nutrient agar slopes incubated for 24 hr at 37° and then kept at room temperature. Material for examination under the electron microscope was harvested from nutrient agar cultures incubated at 37° for 24 hr. The bovine vibrios were subcultured at monthly intervals in Brewer's medium incubated for 5 days at 37° and then left at room temperature. Cultures for examination were grown on blood agar containing 0·1% (w/v) of sodium thioglycollate (Morgan, 1958) and incubated for 5 days at 37° in an atmosphere of 10% (v/v) CO_2 in air.

Electron microscopy. The organisms were suspended in distilled water, mounted on grids and examined according to the method described by Grace (1954).

RESULTS

The three cultures of *V. fetus* showed some straight and curved organisms, many S shapes and short spirals, and a few long spirals. The straight and curved organisms had single polar flagella (Plate 1, f), whereas the S shapes and spirals often had one flagellum at each pole (Plate 1, c, d and e). The morphology of the aerobic vibrios was similar (Plate 1, a). The cultures of nonpathogenic bovine vibrios consisted of curved bacteria with single polar flagella (Plate 1, g and h). Occasionally S shapes were seen. These were either incompletely separated daughters of a parent organism or merely two independent bacteria which had come to lie end to end so as to give them this appearance. No spiral forms were observed. The cultures of *V. fetus* and the aerobic vibrios frequently contained spherical forms (Plate 1, b), which were possibly protoplasts or, more correctly, spheroplasts (Pitzurra & Szybalski, 1959); but these were never observed in cultures of the nonpathogenic bovine vibrios.

DISCUSSION

The identification of *Vibrio* species based solely on the presence of curved organisms does not appear to be satisfactory since many strains of bacteria which are obviously *Pseudomonas* may also exhibit varying degrees of curvature (Pribram, 1929; Breed, Murray & Smith, 1957). The cultures of aerobic vibrios examined in this work all displayed typical curved organisms but, in addition, many S shaped, spiral and some straight forms were present. Henrici (1925), working with *V. cholerae*, also reported this wide range of morphological types within a single culture. It would appear, from these observations, that the presence of S shaped and short spiral organisms is a more reliable criterion for identifying a strain as a *Vibrio* species than is the mere presence of curved forms. The finding that *V. fetus* displays a morphology similar to that of the aerobic vibrios would suggest that it is correctly classified with them.

The spontaneous production of spheroplasts has been noted as a characteristic of both *V. cholerae* (Henrici, 1925; Mackie, 1929) and *V. fetus* (Smith & Taylor, 1919; Plastridge, 1955; Reich, Dunne, Bortree & Hokanson, 1957), while Jeynes (1957) succeeded in causing *V. cholerae* to multiply in this form. All the aerobic vibrio and *V. fetus* cultures examined contained some, apparently naturally occurring, spheroplasts, which would serve to emphasize that these organisms are related.

Though not growing under normal aerobic conditions, *V. fetus* has undoubtedly an obligate requirement for oxygen (Kiggins & Plastridge, 1956). Its basically aerobic nature is further manifested by the ability to produce catalase (Bryner & Frank, 1955; Morgan, 1956) and by the key position which Alexander (1957) has shown the tricarboxylic acid cycle to hold in the organism's metabolism, a characteristic of strict aerobes (Stanier, 1954). The observation that the aerobic vibrios will grow perfectly well under conditions in which *V. fetus* grows (Park, unpublished observation), taken in conjunction with the fact that the growth of *V. cholerae* is improved by higher concentrations of CO_2 than are present in the atmosphere (Jennings & Linton, 1944), further serves to indicate that *V. fetus* is correctly classified as a *Vibrio* species.

The nonpathogenic bovine vibrios are obviously morphologically distinct from the aerobic vibrios and *V. fetus* (see also Morgan, Melrose & Stewart, 1958). Furthermore, they do not give rise to spheroplasts in normal cultures, they are capable of growth under anaerobic conditions (Morgan *et al.* 1958) and they do not produce catalase (Bryner & Frank, 1955; Morgan, 1956). These differences suggest that the nonpathogenic bovine vibrios should not be classified as *Vibrio* species.

My thanks are due to Professor J. F. D. Shrewsbury and Dr. K. A. Bisset for their helpful advice, and to Mr. C. C. Newton for the thoroughness of his technical assistance with the electron microscope.

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Explanation of Plate

Electron micrographs of vibrios.

(a) *V. cholerae* (NCTC 4693), from 24 hr old culture at 37° on nutrient agar, showing rod shaped, curved, S shaped and short spiral cells; ×4,000.

(b)–(h) From 5 day old cultures grown on thioglycollate blood agar at 37° in a 10% CO_2 in air atmosphere.

(b) *V. fetus* (EXT-2), spherical form; ×8,000.

(c) and (d) *V. fetus* (EXT-2), S shaped cells with a flagellum at each pole; ×8,000.

(e) *V. fetus* (EXT-2), short spiral cell with flagellum at each pole; ×8,000.

(f) *V. fetus* (EXT-2), comma shaped cell with flagellum at one pole only; ×8,000.

(g) and (h) Nonpathogenic bovine vibrio DT, showing the curved cells of which the culture solely consisted; ×10,000.

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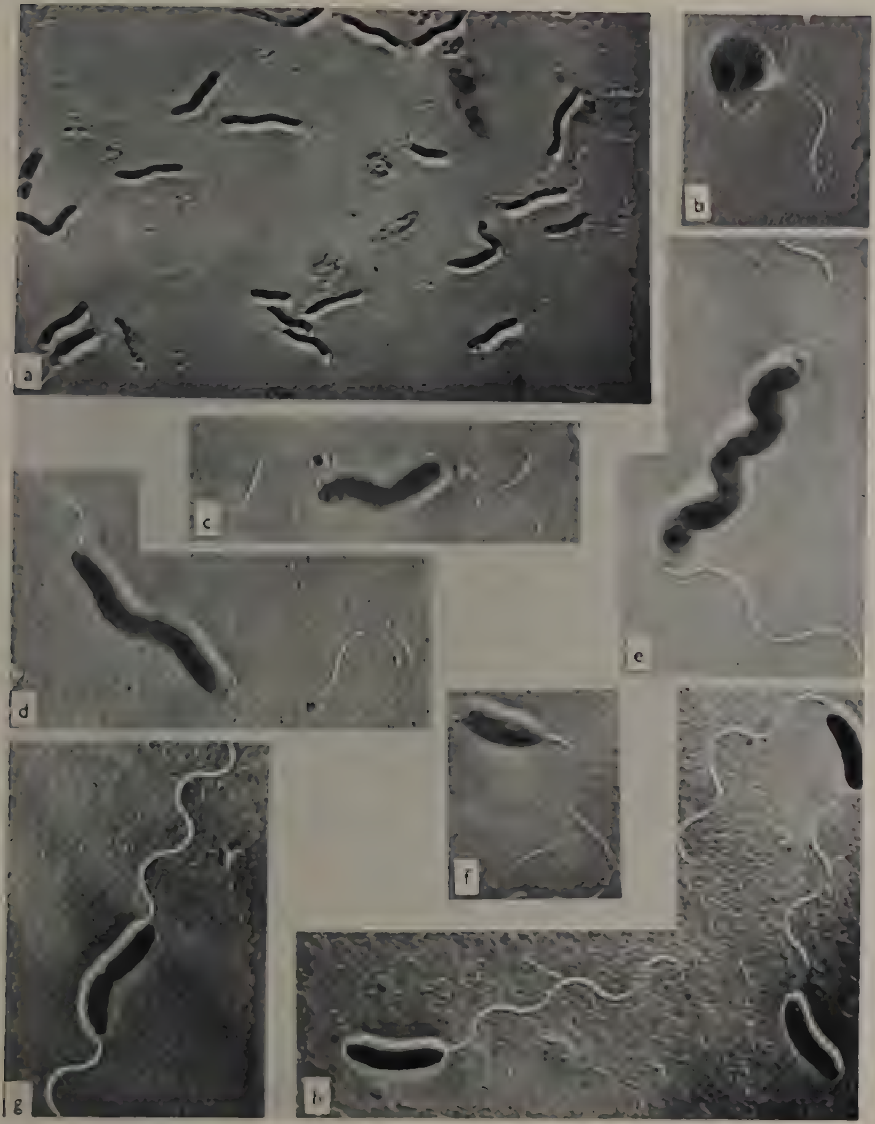

R. W. A. PARK — A NOTE ON THE SYSTEMATIC POSITION OF *VIBRIO FETUS*. PLATE 1

Eddy, B. P. (1961). *J. appl. Bact.* **24**, 27–41.

THE VOGES-PROSKAUER REACTION AND ITS SIGNIFICANCE: A REVIEW

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1. Introduction

The ability of bacteria to produce acetylmethylcarbinol from glucose, and the Voges-Proskauer (V-P) reaction which is used to demonstrate it, are commonly included in descriptions of new organisms and are frequently used for purposes of classification and routine identification. This is particularly true of the coli-aerogenes bacteria where a positive or negative V-P reaction is one of the main diagnostic criteria in the IMViC reactions. Acetylmethylcarbinol can, however, be metabolized by some bacteria, either by reduction or by oxidation, and the quantity remaining to react in the V-P test may represent only a small proportion of the substrate which has actually been metabolized along this particular pathway. Furthermore, the use of very sensitive methods has revealed that differences between bacteria, previously regarded as qualitative, may be only quantitative. It seemed appropriate, therefore, to review the significance of the V-P reaction in the light of present knowledge of the mechanisms of acetylmethylcarbinol production and metabolism and to consider whether the methods now employed are, in fact, the most suitable.

2. The Voges-Proskauer Reaction

Voges & Proskauer (1898) observed that cultures of some bacteria, but not *Bacterium coli communis* (*Escherichia coli*), when made alkaline with potassium hydroxide for absorption of carbon dioxide, developed a red, fluorescent colouration on standing for 24 hr. This red colouration, described by the authors as the *Kalilauge-rothe-reaction* (KOH-red-reaction), constitutes the V-P reaction (*sensu stricto*). Similar observations were made by Durham (1901), Howe (1904) and MacConkey (1905). The last mentioned used the test to differentiate intestinal bacteria, *B. lactis aerogenes* (*Aerobacter aerogenes*) and related types being positive and the remainder, forming the large majority, being negative.

Harden & Walpole (1906) found a discrepancy between the amount of glucose disappearing during fermentation by *Aer. aerogenes* and the amount which could be accounted for in terms of the acids, alcohol and gas which constituted the fermentation products of *E. coli*. They showed that the discrepancy was due to the production by *Aer. aerogenes* of 2,3-butylene glycol (2:3-butanediol) accompanied by smaller quantities of acetylmethylcarbinol (acetoin). Acetoin had previously been shown to be produced by *Bacillus tartricus* (Grimbert, 1901) and by *B. mesentericus*, *B. subtilis* and *Tyrothrix tenuis* (Desmots, 1904).

Harden (1906) showed that whereas neither 2:3-butanediol nor acetoin would give the V-P colouration with alkali alone, acetoin did so in the presence of peptone. The production of the colour at the surface of the liquid suggested to Harden that oxidation was involved; he tried diacetyl and obtained a very deep red colour. Harden & Norris (1911) showed that the diacetyl reaction was given in the presence of compounds with the general formula $NH:C(NH_2)NHR$, i.e. compounds containing the guanidine grouping, for example arginine, agmatine, creatine, dicyanamide and guanidineacetic acid. Differences in the effectiveness of guanidine containing compounds suggested that the constitution of R was important.

Lemoigne (1913, 1920, 1923) demonstrated the ability of certain organisms of the *B. proteus* (*Proteus vulgaris*) and *B. subtilis* groups to carry out the 2:3-butanediol fermentation; he oxidized the 2:3-butanediol and acetoin to diacetyl with ferric chloride, distilled off the diacetyl and prepared the sparingly soluble nickel dimethylglyoxime.

The original V-P reaction comprised the development of a red, fluorescent colouration when a culture was allowed to stand for 24 hr after being made alkaline, but following the work of Harden and his co-workers the reaction was seen to have two features: first, the production and detection of acetoin as a useful test in identification and, second, the ability of bacteria to carry out a 2:3-butanediol fermentation as a taxonomically significant character. The first aspect led to attempts to increase the usefulness of the reaction by making it sensitive to as small amounts of acetoin as possible and by reducing the time before the results of the test could be read. The methods employed fall into three categories: (1) maintaining a high redox potential in the growing culture so that less acetoin is reduced to 2:3-butanediol; (2) promoting the oxidation of acetoin to diacetyl in the test with oxidizing agents; and (3) intensifying the colour produced by the reaction of diacetyl with guanidine containing substances.

Some bacteria give a positive reaction when grown on sucrose but not on glucose, and this was attributed by O'Meara (1931) to the fact that, unlike glucose, sucrose does not lower the redox potential of the medium: therefore less acetoin is reduced to 2:3-butanediol. Because not all fermentative bacteria are able to utilize sucrose, O'Meara tried combinations of glucose and various hydrogen acceptors—potassium nitrate, sodium fumarate, sodium aspartate and methylene blue. The most successful was sodium fumarate. The use of media containing fumarate for the

V-P test has not been adopted generally but is still recommended by some authors (e.g. Dowson, 1949). O'Meara also showed that when a culture gave a weaker reaction after 48 hr than after 24 hr, the intensity of the reaction could be restored to, and even increased above, the 24 hr level by adding a few drops of '10 vol.' H_2O_2 followed by 24 hr further incubation. This can be assumed to be due to oxidation of 2:3-butanediol back to acetoin following a raising of the redox potential.

Levine, Weldin & Johnson (1917), using a basal peptone-phosphate medium, compared the effects of various glucose concentrations (0·5, 1, 2 and 5%), times of incubation (1, 2, 3 and 5 days) and reaction times after the addition of equal volumes of 10% KOH (5, 24, 48 and 72 hr). They found that above 1% glucose the medium was too highly coloured (presumably due to autoclaving) for the reaction to be read, that at 37° all positive cultures reacted after 1 day's incubation, and that, of 140 strains, 92·9% gave a positive reaction within 5 hr of the addition of KOH and the remaining 7·1% within 24 hr. Using sucrose as carbon source, because it imparted no colour to the medium and gave a more intense reaction, they attempted to hasten the colour production by using aerated KOH, and by adding potassium dichromate, potassium chlorate, potassium perchlorate, barium peroxide, calcium hypochlorite and hydrogen peroxide. The most satisfactory were potassium dichromate and hydrogen peroxide used as follows: for potassium dichromate, an equal volume of 10% KOH containing 0·2% of $K_2Cr_2O_7$ was added to the culture and the mixture heated in a boiling water bath for 2 min—a strong reaction was observed within 15 min; for hydrogen peroxide, 3 ml of culture were heated with an equal volume of 10% KOH in a boiling water bath for 2 min and 2–3 drops of H_2O_2 added—the red colour appeared in 1–2 min.

Bedford (1929) added 10 mg of sodium peroxide and 1 ml of 40% KOH to 2·5 ml of culture; the mixture was heated in a boiling water bath for 1 min and then vigorously shaken—the colour appeared in less than 1 min.

Werkman (1930) criticized the use of strong oxidizing agents because of the transience of the colour produced. He obtained a stable, intensified colour by adding 2 drops of 2% $FeCl_3$ and 5 ml of 10% NaOH to 5 ml of culture and shaking the mixture.

O'Meara (1931) considered that oxidizing reagents gave inconstant and unreliable results and tried increasing the concentration of the guanidine containing substance. He added a knife-point (*c.* 25 mg) of creatine followed by 5 ml of 40% NaOH to 5 ml of culture in his synthetic fumarate medium: on shaking a red colour appeared in 2 min. He claimed that 50 p/m of acetoin could be detected with ease and 20 p/m just recognized by this method.

Dorner & Hellinger (1935) found that a combination of the methods of Werkman and O'Meara gave more and clearer positives. To 1 ml of culture were added 1 drop of 1% creatine, 1 drop of 2% $FeCl_3$ and 1 ml of 20% NaOH: the tube was shaken after each addition and incubated at 37°. Levine, Epstein & Vaughn (1934; cited in Manual, 1957), used a solution of 0·3% of creatine in 40% KOH as test reagent: but this solution is unstable.

Barritt (1936) obtained a substantial increase in the sensitivity of the V-P reaction, without any loss of specificity, by using α-naphthol which apparently combines with the product of the reaction of diacetyl with the guanidine compound. Using peptone to supply the guanidine compound the detectable concentration of acetoin was decreased from 20 to 1 p/m and with creatine from 10 to 0·2 p/m. The technique recommended was to add 0·6 ml of 5% α-naphthol in absolute alcohol and 0·2 ml of 40% KOH to 1 ml of culture (3 days at 37°) in glucose phosphate broth and to shake. Positive reactions appeared in 2 to 5 min, the maximum colouration developing within 1 hr. Negative cultures occasionally had a faint pink colour, the reason for which was unknown: characteristically they developed a coppery colour after standing for more than 1 hr, due to the action of KOH on α-naphthol. Barritt obtained positive results with coli-aerogenes bacteria and also with *Brucella melitensis*, *Br. abortus* (bovine), *Staphylococcus aureus*, *Staph. albus*, *Staph. citreus*, *Streptococcus faecalis*, *Vibrio metchnikovi*, the Finkler-Prior vibrio and strains of *V. cholerae* and *Corynebacterium diphtheriae*.

Vaughn, Mitchell & Levine (1939; cited by Batty-Smith, 1941) compared the methods of O'Meara (1931) and Barritt (1936). They found that the Barritt test gave more positive reactions, but might give false positives if the tubes were allowed to stand over 1 hr and that more positives were obtained after incubation at 30° than at 37°. Batty-Smith (1941) also compared the methods of O'Meara and Barritt, but modified the Barritt method as follows. Creatine (*c*. 25 mg) and 2 drops of 2% $FeCl_3$ were added to 5 ml of culture and the mixture lightly shaken. Then 3 ml of 5% α-naphthol in absolute alcohol were added, followed by 1 ml of 40% KOH, and the mixture vigorously shaken for 5 min. Batty-Smith concluded that the results from the two tests were comparable when, after the addition of the reagents, the tubes were left standing long enough for all the positive reactions to develop. He did not confirm the view of Vaughn *et al.* (1939) that the Barritt test gave false positives.

Coblentz (1943; cited in Manual, 1957) reduced the time required for the test by using a massive inoculum and incubating for 6 hr. As test reagents he used α-naphthol (Barritt, 1936) and 0·3% creatine in 40% KOH (Levine *et al.* 1934).

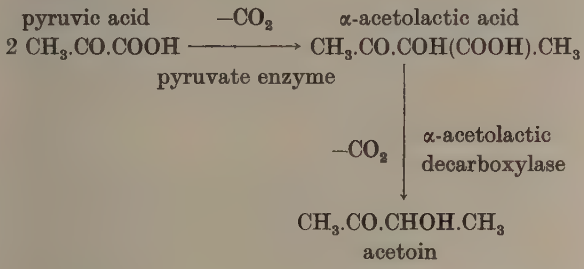
Microtests for the detection of acetoin production have been devised by Fabrizio & Weaver (1951) and by Clarke & Cowan (1952).

With some bacteria the V-P reaction may weaken with prolonged incubation and with others may even become negative. This is probably due to the partial or total conversion of acetoin into compounds which do not give a positive V-P reaction; for example, by reduction to 2:3-butanediol (Table 1) or by oxidation to diacetyl followed by further metabolism.

3. The Production of Acetoin

Silverman & Werkman (1941) obtained a cell-free preparation from *Aer. aerogenes* which was able to produce acetoin from pyruvic acid, two molecules of pyruvic acid giving one molecule of acetoin and two molecules of carbon dioxide. The nature of the intermediate steps in the reaction gave rise to some controversy. One school of thought believed that at least one molecule of pyruvic acid was decarboxylated to acetaldehyde before condensation took place: the other held that the first compound to be produced was α-acetolactic acid. Berg & Westerfeld (1944), for example, considered that in muscle, decarboxylation to acetaldehyde, which may not appear in the free state, must precede condensation. This view was supported by the results of Gross & Werkman (1947) using C^{13} labelled acetaldehyde and yeast enzyme. Watt & Krampitz (1947), however, suggested that α-acetolactic acid is an intermediate in acetoin formation by *Staph. aureus*. Vennesland (1951) considered that, in view of the results of Berg & Westerfeld and Gross & Werkman, this suggestion should be regarded with reservation. The background to these differences has been reviewed in detail by Ochoa (1951).

The controversy was resolved by Juni (1952*a*) who demonstrated two enzymes in extracts of *Aer. aerogenes*, one (stimulated by co-carboxylase) producing α-acetolactic acid from pyruvic acid and the other decarboxylating α-acetolactic acid to acetoin but having no action on pyruvic acid. Extracts from bacteria unable to produce acetoin (e.g. *E. coli*, *Micrococcus lysodeikticus* and *Rhodospirillum rubrum*) were unable to decarboxylate α-acetolactic acid, whereas this reaction occurred with extracts from acetoin producing bacteria (e.g. *B. subtilis*, *Staph. aureus* and *Serratia marcescens*). Juni did not show whether or not extracts from V-P negative bacteria were able to produce α-acetolactic acid from pyruvic acid. The results of fractionation experiments by Happold & Spencer (1952*b*) suggest that Juni's pyruvate enzyme may not be a single entity.



$$\begin{tikzcd}
\overset{\text{pyruvic acid}}{2\,CH_3.CO.COOH} \arrow[r, "-CO_2", "\text{pyruvate enzyme}"'] & \overset{\text{α-acetolactic acid}}{CH_3.CO.COH(COOH).CH_3} \arrow[d, "-CO_2"', "\text{α-acetolactic decarboxylase}"] \\
 & \underset{\text{acetoin}}{CH_3.CO.CHOH.CH_3}
\end{tikzcd}$$

From comparative studies of the production of acetoin by bacteria, yeast and muscle, Juni (1952*b*) concluded that there are at least three mechanisms of acetoin formation. That in bacteria has α-acetolactic acid as an intermediate; those in yeast and muscle, which themselves are not identical, do not. The α-acetolactic acid pathway of acetoin formation was also found in *Strep. faecalis* by Dolin & Gunsalus (1951), but De Ley (1959) found both the acetaldehyde and α-acetolactic acid pathways occurring side by side in *Acetobacter rancens*, *A. pasteurianus* and *A. ascendens*. Watt & Werkman (1951) established that *Staph. aureus* is able to metabolize pyruvate in two ways and that the acetoin producing system is only developed if acid is formed during growth.

It is clearly desirable to know how much of the substrate has been metabolized along the acetoin pathway since only then can it be decided whether or not the acetoin producing mechanism constitutes a major metabolic pathway. To do so it is necessary to take into account the products into which acetoin may be converted in a bacterial culture.

4. The Further Metabolism of Acetoin

Acetoin can be metabolized either by reduction to 2:3-butanediol which accumulates unless reoxidation takes place or, apparently less commonly, by oxidation to diacetyl which may be further metabolized. Walpole (1911) found that *Aer. aerogenes* was able to produce acetoin from 2:3-butanediol, more acetoin being produced if the culture was aerated. Stahly & Werkman (1942) observed that in oxygenated cultures of *B. polymyxa* large quantities of the glycol accumulated while the E_h remained low, but part became oxidized to acetoin when the E_h rose to a fairly high level. Little acetoin accumulated during the early stages of fermentation and added carbinol was reduced to glycol, but only while the E_h remained low (Fig. 1). They concluded that 2:3-butanediol and acetoin constitute a reversible oxidation-reduction system. In these experiments only about half the glucose fermented was recovered as acetoin plus 2:3-butanediol and in every case the final concentration of glycol was higher than that of carbinol.

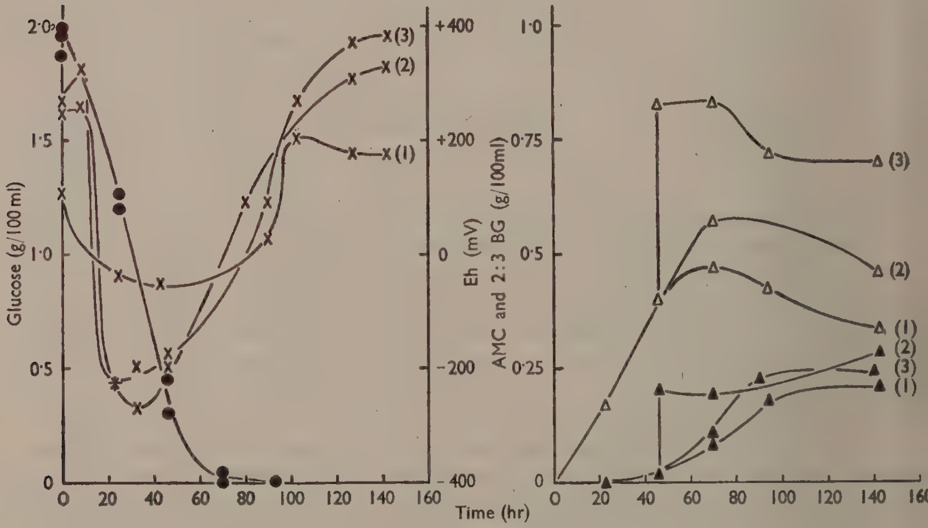


Fig. 1. Changes in oxidation-reduction potential and concentrations of glucose, acetylmethylcarbinol (AMC) and 2:3-butylene glycol (2:3 BG) during the fermentation of glucose by *Bacillus polymyxa*. (1), without addition; (2), acetylmethylcarbinol added after 46 hr; (3), 2:3-butylene glycol added after 46 hr. (Data from Stahly & Werkman, 1942). Circles, glucose; full triangles, acetylmethylcarbinol; crosses, E_h; open triangles, 2:3-butylene glycol.

The interconvertibility of acetoin and 2:3-butanediol was shown rather more convincingly by Happold & Spencer (1952*a*) using washed suspensions of *Aer. aerogenes* (Fig. 2). With glucose as substrate, production of glycol predominated until about two thirds of the glucose had been used; then the carbinol increased at the expense of the glycol and the concentration of the latter fell almost to zero. With pyruvate as substrate, carbinol predominated throughout the experiment, which may be due to the fact that pyruvate does not reduce the O-R potential as does glucose.

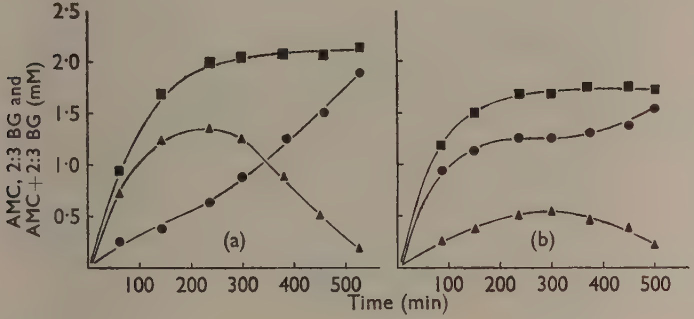


Fig. 2. Progress curves for acetylmethylcarbinol (AMC) and 2:3-butylene glycol (2:3 BG) production by washed cell suspensions of *Aerobacter aerogenes* from (a) glucose and (b) pyruvate. (Reproduced, with permission, from Happold & Spencer, 1952*a*).
Circles, acetylmethylcarbinol; triangles, 2:3-butylene glycol; squares, acetylmethylcarbinol+2:3-butylene glycol.

Table 1 shows the accumulation of 2:3-butanediol and acetoin during the anaerobic (cf. Stahly & Werkman, 1942) fermentation of glucose by *Aer. indologenes* (Reynolds & Werkman, 1937*a*): under the conditions of this experiment all the acetoin was ultimately reduced to glycol and there was no indication of any reversal. Table 2 shows the proportions of acetoin and 2:3-butanediol found by various workers using different organisms and substrates. *Staph. aureus* is the only organism listed which shows an excess of acetoin over 2:3-butanediol and its substrate was pyruvic acid

Table 1. *The accumulation of acetoin and 2:3-butanediol during the fermentation of glucose by* Aerobacter indologenes

(Data from Reynolds & Werkman, 1937*a*)

Time (hr)	Glucose fermented (mMoles/l.)	Acetoin (mMoles/l.)	2:3-Butanediol (mMoles/l.)
24	14·33	0·266	6·02
28	32·70	0·452	13·87
31	36·10	0·78	16·70
35	41·00	0·452	22·30
47	61·10	1·22	35·45
64	82·50	0·452	49·8
81	100·30	0·11	66·05
110	123·80	0·89	79·40
209	127·30	0·0	84·25

(cf. Happold & Spencer, 1952*a*). However, *Staph. aureus* is not a strongly fermentative species and the present writer has observed that cultures of some staphylococci (*sensu* Shaw, Stitt & Cowan, 1951) in glucose phosphate peptone water smell strongly of diacetyl, presumably derived from the oxidation of acetoin.

Table 2. *The proportions of acetoin and 2:3-butanediol found during the fermentation of various carbon sources by different bacteria*

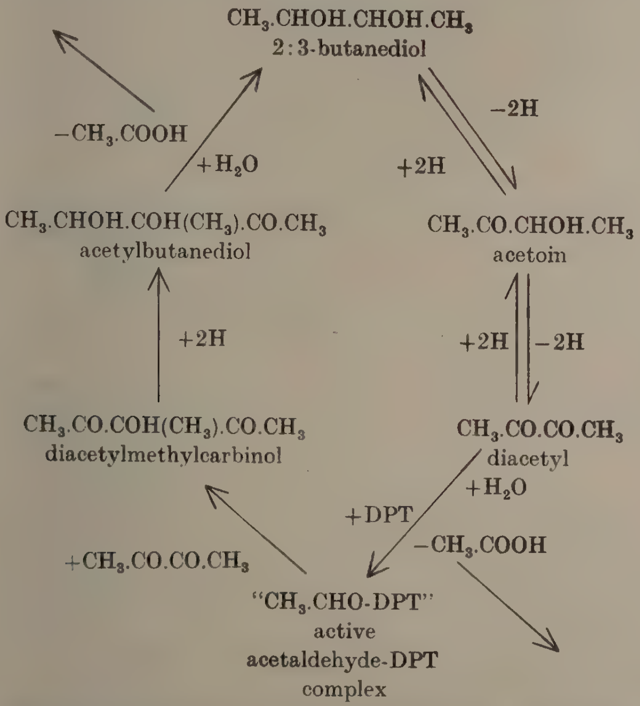
Organism	Substrate	Acetoin (g/100 g substrate utilized)	2:3-Butanediol (g/100 g substrate utilized)	Reference
Clostridium acetobutylicum	Glucose	2·4	Not determined	Donker (1926)
Bacillus macerans	Glucose	trace	0·6	
Bacillus polymyxa	Glucose	trace	19·1	
Aerobacter faeni	Xylose (aerobic)	2·6	10·6	Breden & Fulmer (1931)
	Xylose (anaerobic)	0·25	13·7	
	Sucrose (aerobic)	6·4	15·1	
	Sucrose (anaerobic)	0·6	16·7	
*Aeromonas hydrophila**	Glucose	0·83	27·4	Stanier & Adams (1944)
	Xylose	1·5	23·4	
	Pyruvic acid	0·5	3·6	
Staphylococcus aureus	Pyruvic acid	16·7	6·1	Watt & Werkman (1951)

* Because this organism fermented xylose it is improbable that it was an *Aeromonas* (Eddy, 1960).

Brewer, Michaelian, Werkman & Hammer (1937) reported that the concentration of diacetyl in cultures of citric acid fermenting streptococci and in butter cultures was increased several-fold by bubbling air through them at up to 60 lb/in^2. Slight increases in acetoin concentration were also found. Sebek & Randles (1952) found one strain of *Pseudomonas fluorescens* and two of *Ps. aeruginosa* to be capable of oxidizing 2:3-butanediol through acetoin to diacetyl which was then further metabolized. Strecker & Harary (1954) isolated two enzymes from *Aer. aerogenes* and *Staph. aureus*, one catalyzing the reversible oxidation of 2:3-butanediol to acetoin (2:3-butanediol dehydrogenase) and the other catalyzing the reduction of diacetyl to acetoin (diacetyl reductase). Both enzymes were shown to be DPN linked.

Paine (1927) found that strains of *Aer. aerogenes*, and also some strains of V-P negative bacteria, were able to destroy acetoin. Williams & Morrow (1928) obtained similar results with strains of *Aer. aerogenes*, *B. subtilis*, *B. cereus*, *B. mycoides* and *B. vulgatus*, with three other sporeformers, and with 71 green fluorescent types, but not with strains of *Salmonella*, *Eberthella*, *Proteus* and *Serratia*. They concluded that acetoin was probably being used as a source of carbon. This was confirmed by Tittsler (1938) who observed that strains of *Aer. aerogenes* and *Aer. oxytocum* were able to produce acid and gas in a synthetic medium containing acetoin as sole carbon source. This behaviour may explain transiently positive V-P reactions and those which become weaker as incubation is prolonged.

Juni & Heym (1956*a*,*b*, 1957) postulated a cyclic pathway for the dissimilation of 2:3-butanediol, acetoin and diacetyl by bacteria able to use 2:3-butanediol and acetoin as sole sources of carbon, e.g. *Aer. aerogenes*, *Micrococcus ureae* and *Ps. fluorescens*. In the cycle, 2:3-butanediol is oxidized first to acetoin and thence to diacetyl. The diacetyl is split with the addition of diphosphothiamine (DPT) and water to give acetic acid and an active acetaldehyde-DPT complex. This latter then reacts with a further molecule of diacetyl giving diacetylmethylcarbinol and free DPT. The diacetylmethylcarbinol is reduced to acetylbutanediol which is hydrolyzed to acetic acid and 2:3-butanediol. Thus two molecules of 2:3-butanediol give one molecule of regenerated 2:3-butanediol and two molecules of acetic acid. It is not known how many distinct enzymes are involved in the cycle, since the 2:3-butanediol dehydrogenase is relatively unspecific and differs according to whether the cells are grown on glucose or acetoin as carbon source.


$CH_3.CHOH.CHOH.CH_3$
2:3-butanediol
$-2H$
$+2H$
$-CH_3.COOH$
$+H_2O$
$CH_3.CHOH.COH(CH_3).CO.CH_3$
acetylbutanediol
$CH_3.CO.CHOH.CH_3$
acetoin
$+2H$
$+2H$
$-2H$
$CH_3.CO.COH(CH_3).CO.CH_3$
diacetylmethylcarbinol
$CH_3.CO.CO.CH_3$
diacetyl
$+H_2O$
$+DPT$
$-CH_3.COOH$
$+CH_3.CO.CO.CH_3$
"$CH_3.CHO$-DPT"
active
acetaldehyde-DPT
complex

When considering whether or not the acetoin producing mechanism constitutes a major metabolic pathway for any organism it is necessary to take into account the products of further metabolism. In many cases there will be solely 2:3-butanediol but in some cases, depending upon the oxidation-reduction conditions and the presence of the necessary enzymes, diacetyl and derivatives from it may be present.

Fermentation products have usually been given only in terms of acetoin and 2:3-butanediol: the percentages by weight of various substrates appearing as these products are quoted in Table 2. Other values are: *c.* 27% from glucose (Harden & Walpole, 1906), 32% from glucose (Reynolds & Werkman, 1937*a*) and 4% and 16% from glucose and pyruvate respectively (Happold & Spencer, 1952*a*).

5. THE USE OF THE VOGES-PROSKAUER REACTION IN CLASSIFICATION

Many bacteria produce acetoin during growth on carbohydrate containing media but the character has only been used for classification or identification in four groups, viz. *Aeromonas*, *Bacillus*, the coli-aerogenes group and *Staphylococcus*. In the genus *Bacillus* the test is used only for the purpose of species identification (Gibson & Topping, 1938), but in the other three groups the ability to produce acetoin has been used at higher levels of classification.

Voges & Proskauer (1898) noted that *E. coli* did not give their KOH-red reaction, and Durham (1901) found that *Aer. aerogenes* did so but not organisms with 'colon-like' or 'typhoid-like' morphology. MacConkey (1905) observed that the great majority of organisms isolated from faeces, both human and animal, were negative. Levine (1916) showed that among lactose fermenting Gram-negative rods there was a high negative correlation between the results of the V-P and methyl red (MR) tests, *E. coli* being MR + and V-P – and *Aer. aerogenes* being MR – and V-P +. It is believed that when the cultures of *aerogenes*-like organisms become sufficiently acid the acetoin producing mechanism is developed, by which most of the pyruvate is metabolized and the culture loses its acidity, presumably due to the lack of any further production of acid and the destruction of that already produced. Reynolds & Werkman (1937*a*) suggested that in *Aer. indologenes* the acetic acid produced before the development of the acetoin producing mechanism was converted into acetoin and thence to 2:3-butanediol.

Some strains of *aerogenes*-like bacteria which ferment lactose slowly or not at all ('paracolons') fail to lose all their acidity at 37° and give a very weak or negative V-P reaction. At 20°, however, total or partial reversal of acidity takes place and a strong V-P reaction is given. These organisms are placed in the *Hafnia* group. Since these cultures develop the necessary acidity, it seems probable that in this group a temperature of 37° is inimical to the formation of the acetoin producing system (cf. Alford, 1960). In other bacteria, e.g. *Aeromonas liquefaciens* (Caselitz, 1958; Eddy, 1960), a combination of positive MR and V-P reactions of different intensities is not infrequently encountered.

Reynolds & Werkman (1937*b*) found that vigorously aerated cultures of *E. coli* produced small amounts of acetoin and hence possessed the necessary mechanism. However, only after 6 days incubation was the amount of acetoin sufficient to give a positive reaction by O'Meara's (1931) method without first being concentrated tenfold by distillation. Unaerated cultures showed no traces of acetoin.

Segal (1940) suggested that the ability to use acetoin as sole source of carbon might be used to differentiate *Staph. albus* from *Staph. aureus*. He observed that 15 of 18 strains of *Staph. albus* produced "a yellowish turbidity" in Tittsler's (1938) synthetic medium whereas 23 of 26 strains of *Staph. aureus* produced no apparent change. The interpretation of these results is open to question since both *Staph. aureus* and *Staph. albus* have been shown to require for growth thiamin and either nicotinic acid or nicotinamide (Kögl & Wagtendonk, 1938, cited by Elek, 1959; Koser, Finkle, Dorfman, Gordon & Saunders, 1938) and also certain amino-acids, none of which was included in Tittsler's medium.

Shaw, Stitt & Cowan (1951), in attempting to subdivide all catalase +, Gram + cocci into biochemical groups, put all the coagulase -, V-P + strains into one group (i.e. group 2—*Staph. epidermidis*). No other biochemical character was found to give a satisfactory correlation with the V-P reaction, but 93% of the group 2 strains investigated had an optimum temperature of 37° compared with only 62% of the similar, but V-P −, group 3 strains. The authors stressed the arbitrary and artificial nature of this classification of micrococci and pointed out that if very sensitive methods of testing for acetoin (e.g. that of Batty-Smith, 1941) are used the reaction may lose its differential value. Furthermore, Eddy (unpublished data) has found that some staphylococcal strains which give negative V-P reactions in unshaken glucose-peptone water cultures, possibly due partly to poor growth, give positive reactions in shaken glucose-peptone water-yeast extract cultures. Cowan's (1953) remarks concerning testing for the production of hydrogen sulphide by bacteria are clearly apposite here: ". . . if our findings make nonsense of schemes of classification now in use, then we should develop new schemes". Hill (1959), when applying the Adansonian method of classification to the staphylococci, found that the V-P − group 3 (*Staph. lactis*) of Shaw *et al.* (1951) is probably not homogeneous.

Kluyver & van Niel (1936) proposed the name *Aeromonas* for polarly flagellated bacteria which ferment carbohydrate with the production of acid and gas "and 2,3-butylene glycol (?)", i.e. polarly flagellated organisms with a fermentation pattern similar to that of *Aerobacter*. The type species designated was *Aeromonas liquefaciens* Beijerinck (*Aerobacter liquefaciens* Beijerinck). Their reasons for hesitating to specify 2:3-butanediol were probably that Beijerinck's (1900) description of the genus had antedated by some years Harden & Walpole's (1906) demonstration that the characteristic fermentation product of *Aer. aerogenes* is 2:3-butanediol, and that Beijerinck's original isolates were no longer available for study.

Miles & Miles (1951), having studied a number of presumptive strains of *Aeromonas*, accepted the idea that the qualifying criterion should be production of 2:3-butanediol and suggested that if its production proved to be " . . . . a property characterizing an otherwise substantially homogeneous group within the Pseudomonadaceae, the genus *Aeromonas* Kluyver & van Niel should be accepted". Eddy (1960), on the other hand, considered that the genus *Aeromonas* should comprise three species of which only one, *Aeromonas liquefaciens*, is V-P + and that in this species, as in *Aerobacter*, a positive V-P reaction is sufficient evidence of the existence of a metabolic pathway leading to 2:3-butanediol.

Gibson & Topping (1938) distinguished *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus* and *B. anthracis* from *B. megaterium* and the *B. circulans* group, and *B. polymyxa* from *B. macerans*, on the basis of the V-P reaction, in each case the former being positive. Donker (1926), however, showed that *B. macerans* produced traces of acetoin and small amounts of 2:3-butanediol. Eddy (unpublished data) has compared the reactions given by strains of *Bacillus* spp. in static and shaken culture and found that whereas *B. licheniformis*, *B. polymyxa* and *B. pumilus* are positive and *B. megaterium* is negative (cf. Lemoigne, Delaporte & Croson, 1944) under both conditions, *B. circulans* and *B. macerans* give negative reactions in static culture but weakly positive reactions in shaken culture. Hence the differentiation adopted by Gibson & Topping (1938) is valid only under specified cultural conditions. Smith, Gordon & Clark (1952) compared the effects of cultural and test conditions on the V-P reaction given by some species of *Bacillus*.

6. DISCUSSION

There are clearly two aspects involved. The first is the use of acetoin production as a taxonomically important feature; the second is the use of the V-P reaction or a modification of it as a routine test in bacterial identification.

Since acetoin is the first 4-carbon compound to be formed, and since it can be metabolized by simple reduction or oxidation changes involving the addition or removal, respectively, of 2 H atoms, the important taxonomic character is the ability of bacteria to carry out the reactions leading to the formation of acetoin. The conversion of the acetoin, once formed, into other products clearly depends largely upon external conditions. There is therefore no justification for postulating the production of 2:3-butanediol as the taxonomically important character (Kluyver & van Niel, 1936; Miles & Miles, 1951). If it is the case that acetoin can be formed by some bacteria by two different pathways (De Ley, 1959), these must constitute two distinct taxonomic features irrespective of the identity of the end product.

Provided there is no loss of specificity, the most sensitive methods available should be employed when carrying out the V-P test (Cowan, 1953) as although a substantial fraction of the substrate may have passed down this reaction chain (Tables 1 and 2), the larger part of the acetoin produced may have been converted to 2:3-butanediol which does not give the V-P reaction. Indeed there are occasions when all the acetoin may be converted into 2:3-butanediol and a false negative obtained (Reynolds & Werkman, 1937*a*; Murphy, Muirhead, Watson & Barnwell, 1951). Hence it would be of value if a satisfactory method of carrying out the V-P test could be found in which either the reduction of acetoin to 2:3-butanediol during fermentation was suppressed or in which both acetoin and 2:3-butanediol were oxidized to diacetyl when the test was performed. This idea is contrary to that of Werkman (1930) who, discussing the use of oxidizing agents, remarked that "Ferric chloride will not result in the oxidation of 2,3-butylene glycol under the conditions of the test, and any organism which may have reduced the acetylmethylcarbinol to 2,3-butylene glycol will not give a false V-P reaction". In view of the mechanism of acetoin and 2:3-butanediol production, it is the present writer's opinion that false negatives are more likely to occur than false positives. Indeed a positive reaction, in the sense suggested, could only be false if some substance not related to diacetyl were responsible which seems unlikely (Barritt, 1936).

The chemical oxidation of the glycol to the carbinol requires strong oxidizing agents (Happold & Spencer, 1952*a*) and seems of doubtful applicability to the routine

V-P test, especially in the presence of other oxidizable constituents of the culture. The most suitable modifications of the present technique would seem to be: (a) raising the O-R potential, after fermentation has ceased (cf. the use of aerated cultures by Stahly & Werkman, 1942), either by adding hydrogen peroxide (O'Meara, 1931) or by aeration (e.g. shaking); (b) poising the O-R potential at a high level during fermentation by the addition of a hydrogen acceptor such as fumarate (O'Meara, 1931); and (c) replacement of glucose by some C-source such as pyruvate (Happold & Spencer, 1952*a*) which will not reduce the O-R potential to a low level during fermentation.

In V-P negative cultures, the absence of 2:3-butanediol can be confirmed by extracting with ether, removing the ether by distillation, taking up the residue in water, spotting on paper and spraying with a solution of lead tetra-acetate in benzene (Eddy, 1960). If a 2:3-diol such as 2:3-butanediol is present to act as a reducing agent, light spots appear against the dark background on the paper caused by decomposition of the unreduced reagent.

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A STUDY OF ANTIGENS OF THE AEROBIC SPOREFORMING BACTERIA

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SUMMARY: Spore, vegetative O and vegetative H agglutinogens were demonstrated as separate entities in *Bacillus* species. Spore precipitinogens were distinct from those of the vegetative stage, the latter being associated with the H antigen.

Injection of viable spores into rabbits resulted in the formation of antibodies for the vegetative phase as well as spore antibodies, while injection of autoclaved spores gave rise to spore antibodies only.

Spore agglutinogens varied in their strain specificity, *B. cereus* and *B. megaterium* especially showing much subspecies specificity, but the spore precipitinogens showed a high degree of species specificity and could be of considerable taxonomic value. The multiplicity of spore precipitinogens in certain species was demonstrated and inter-species cross reactions were studied.

The spore, somatic and flagellar antigens of *Bacillus polymyxa* were studied by Davies (1951) using the agglutination technique. The three antigens proved to be serologically distinct and there was a species specific antigen present in the spores of all 39 strains studied. Lamanna (1942) and Doak & Lamanna (1948) showed that the spores and vegetative cells of several *Bacillus* species possessed precipitinogens and that the spore precipitinogens were of some taxonomic value.

The aims of the present work were to extend Davies' observations to other members of the genus, to include a study of spore and vegetative cell precipitinogens and to try to assess the taxonomic value of the different types of antigen.

Materials and Methods

Preparation of antigens

Whether intended for injection into rabbits or for use in agglutination or precipitation tests, only freshly prepared antigens were used and all cultures were checked microscopically for purity.

Vegetative cell antigens

Cultures were grown in nutrient broth (Oxoid) in 4 oz McCartney bottles. These were inoculated with a loopful of an overnight broth culture and incubated at 30° for 12 hr with continuous gentle shaking. The cultures were centrifuged and the cells washed 6 times in 25 ml amounts of sterile saline. The final deposit was resuspended in saline, shaken rapidly for 10 min and centrifuged lightly to throw down cell aggregates. The homogeneous supernatant suspension was pipetted off, diluted with sterile saline to a turbidity equivalent to Brown's opacity tube no. 8 and used for the preparation of vegetative H and O antigens.

H antigen. Formalin, at a final concentration of 0·25% (v/v) was added to the above suspension, which was then incubated for 2 hr at 37° before use. Subsequent storage was at 4°.

O antigen. The vegetative cell suspension was heated in a water bath at 100° for 1 hr and kept at 4°.

Spore antigens

Cultures were grown on nutrient agar (Oxoid) for 10–12 days at 30°. When sporulation had reached a maximum the cultures were harvested into sterile distilled water and washed twice before resuspension in the same medium. In order to free the spores from vegetative cell debris further treatment was necessary. This differed with the different species.

Suspensions of *B. cereus*, *B. megaterium*, *B. subtilis*, *B. licheniformis*, *B. pumilus*, *B. polymyxa*, *B. laterosporus* and *B. coagulans* were incubated for 4 days at 37° in a 1:20,000 solution of thiomersalate, by which time lysis of vegetative material was complete (Delpy & Chamsy, 1949).

Suspensions of *B. alvei*, *B. circulans*, *B. brevis* and *B. sphaericus* spores could not be purified in this way but responded to treatment with a preparation of *B. cereus* lytic principle (Norris, 1957) prepared as follows: an overnight culture of *B. cereus* (strain M.8) grown on nutrient agar (Oxoid) at 30° in a 20 oz Roux bottle was harvested into 10 ml of sterile distilled water and the resulting suspension, after standing for a few minutes, was centrifuged. The supernatant was sterilized by filtration through a cellulose acetate membrane (Oxoid) and 1:20,000 thiomersalate added to give the lytic principle preparation. Deposits from cultures of the 4 species were resuspended in this preparation and incubated at 37° for 2 days, by which time lysis of vegetative material was complete, the spores remaining unchanged as judged by tests for viability and heat resistance.

Spores, prepared by either of these methods, were washed 6 times in sterile distilled water, resuspended in sterile saline solution and shaken vigorously for 10 min. Light centrifugation deposited larger spore clumps and the smooth supernatant suspension was diluted to a turbidity equivalent to Brown's opacity tube no. 8 and stored at 4°. Spore suspensions for the agglutination or precipitation test were used unheated except where otherwise stated. Those used for preparation of antisera were normally autoclaved at 15 lb/in^2 for 20 min.

Preparation of antisera

Antigen suspensions (turbidity equivalent to Brown's opacity tube no. 8) were injected intravenously into adult rabbits at 3 day intervals. Four doses of 0·5, 1·0, 2·0 and 3·0 ml respectively sufficed to produce high titre vegetative cell H antisera and a further 4 doses of 3 ml each were administered for the production of vegetative cell O and spore antisera. Antisera were preserved with 1:10,000 thiomersalate and stored at 4°.

Serological tests

Agglutination tests

These were carried out by the method of Lamanna (1940*a*,*b*). Doubling dilutions of antiserum in saline (0·1 ml amounts) were prepared in $\frac{1}{2} \times 3$ in. test tubes and 0·1 ml of antigen suspension (Brown's tube no. 8) added to each. The tubes were shaken in a water bath at 30° for 45 min, the movement having an amplitude of $2\frac{1}{2}$ in. and a rate of 1 oscillation/sec. Saline (0·8 ml) was then added to each tube and the tests read immediately. Controls, in which the antiserum was replaced by saline or by normal serum, obtained from the same rabbit before injection, were always included.

Agglutinin absorption tests

The absorbing antigen was suspended in saline to give a suspension as concentrated as was convenient to handle (usually antigen suspended in twice its volume of saline). The antiserum to be absorbed (0·1 ml) was mixed with 0·2 ml of the antigen suspension and shaken at 30° for 30 min. A further 0·2 ml of antigen was then added and the shaking repeated. The suspension was then centrifuged and a clear absorbed serum obtained. The method proved to be highly efficient and only rarely was it necessary to repeat the process.

Precipitin tests

Antigen extract. Cells from 5 ml of antigen suspension, as prepared for agglutination, were harvested by centrifugation and extracted with 0·1 ml of formamide at 150° for 15 min. The extract was allowed to cool, 0·25 ml of acid alcohol (95 parts of absolute alcohol and 5 parts of 2N HCl) was added and the mixture well shaken. The precipitate was spun off and the supernatant transferred to a clean tube and 0·5 ml of acetone added. The resulting precipitate was harvested by centrifuging and resuspended in 1 ml of saline. The suspension was neutralized to phenol red with dilute sodium carbonate solution, allowed to stand at room temperature for a few minutes and any undissolved material spun down. The clear supernatant solution constituted the antigen for the test.

Test. Ring tests were performed in 2·5 mm internal diameter glass tubing and observations, at room temperature, taken over a period of 1 hr. Positive reactions normally developed very rapidly, taking the form of a dense white zone of precipitate (sometimes two zones) near the antigen-antiserum interface. Occasionally secondary zones formed after about 15 min within the antiserum layer and at some distance from the interface.

Where no zones developed the test was repeated using antigen solutions diluted 1:5 and 1:10 with saline, but in no instance did this result in a positive reaction replacing a negative one.

RESULTS

The antibody response to living and heat killed spores

Antisera were prepared against viable and heat killed spores of *B. cereus*, *B. subtilis* and *B. alvei* and their reactions compared. Table 1 shows the agglutinin titres and precipitation reactions obtained with different types of antigen for antisera to spores of *B. cereus* M.8, *B. subtilis* 3610 and *B. alvei* 2198. The differences between the first two appear quantitative rather than qualitative, but with the latter a different picture was obtained.

With each of the 3 organisms the injection of unheated spores resulted in the production of both spore and (vegetative) H antibodies, the latter capable of being absorbed by their respective H antigens. Autoclaving the spores before injection prevented the appearance of H antibody, but spore antibodies were still produced.

Spores of *B. cereus* M.8 steamed for 1 hr gave responses similar to those of autoclaved spores.

Table 1. *A comparison of antisera to viable and heat killed spores of* B. cereus *M*.8, B. subtilis 3610 *and* B. alvei 2198

Antiserum to:	Absorbing antigen	Agglutination reactions. Titres* obtained with											
		Autoclaved spores			Unheated spores			Vegetative O antigen			Vegetative H antigen		
		(c)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
Unheated spores	None	2,560	320	320	5,120	320	5,120	0	0	0	2,560	80	1,280
	H antigen	2,560	320	320	5,120	320	320	0	0	0	†	0	0
	Autoclaved spores	0	0	0	0	0	2,560	0	0	0	1,280	80	1,280
	Unheated spores	0	0	0	0	0	0	0	0	0	1,280	80	0
Autoclaved spores	None	5,120	640	640	10,240	640	320	0	0	0	0	0	0
Vegetative H antigen	None	0	0	†	0	0	640	320	160	640	10,240	10,240	20,840
	Unheated spores	0	0	0	0	0	0	320	160	320	10,240	5,120	†

	Precipitation reactions* with extract of					
	Spores			Vegetative cells		
	(a)	(b)	(c)	(a)	(b)	(c)
Unheated spores	+	+	+	+	+	+
Autoclaved spores	+	+	+	−	−	−
Vegetative H antigen	−	−	+	+	+	+

* Titres: (a), *B. cereus* M.8; (b), *B. subtilis* 3610; (c), *B. alvei* 2198.

† A trace of agglutination at a serum dilution of 1:20.

The relationship between antigens from the spore and vegetative stages

This part of the investigation was primarily concerned with *B. cereus*, *B. subtilis*, *B. licheniformis* and *B. alvei*. Antisera were prepared against H and O antigens from the vegetative stage and against autoclaved spores of a representative strain of each of these species and the reactions of these antisera with the different types of antigen were studied. Additional information was sought from a study of vegetative O and of spore antisera prepared against typical strains of a further 8 species.

The results obtained with *B. subtilis* 3610 are shown in Table 2. Those for *B. cereus* M.8 and *B. licheniformis* 2843 were essentially similar. The behaviour of *B. alvei* 2198 is summarized in Table 3. The agglutination titres obtained when spore and vegetative cell O antisera were tested against antigens of the homologous

Table 2. *The relationship between spore and vegetative cell antigens of* B. subtilis 3610

Antiserum to:	Absorbing antigen	Agglutination reactions. Titres obtained with			
		Autoclaved spores	Unheated spores	Vegetative O antigen	Vegetative H antigen
Autoclaved spores	None	640	640	0	0
	Unheated spores	0	0	0	0
	Autoclaved spores	0	0	0	0
Vegetative O antigen	None	0	0	640	0
Vegetative H antigen	None	0	0	160	10,240
	O antigen	0	0	0	10,240

	Precipitation reactions with extract of	
	Spores	Vegetative cells
Autoclaved spores	+	–
Vegetative O antigen	–	–
Vegetative H antigen	–	+

Table 3. *The relationship between spore and vegetative cell antigens of* B. alvei 2198

Antiserum to:	Absorbing antigen	Agglutination reactions. Titres obtained with			
		Autoclaved spores	Unheated spores	Vegetative O antigen	Vegetative H antigen
Autoclaved spores	None	640	320	0	0
	Unheated spores	0	0	0	0
	Autoclaved spores	0	0	0	0
Vegetative O antigen	None	0	0	640	20
	H antigen	0	0	640	0
Vegetative H antigen	None	†	640	640	20,480
	Autoclaved spores	0	640	640	20,480
	O antigen	0	640	0	10,240
	Unheated spores	0	0	320	†

	Precipitation reactions with extract of	
	Spores	Vegetative cells
Autoclaved spores	+	–
Vegetative O antigen	–	–
Vegetative H antigen	+	+

† A trace of agglutination at a serum dilution of 1:20.

organism are given in Table 4 and the reactions observed when spore antisera were examined for precipitins against homologous spore extracts are shown in Table 5.

There was no cross reaction in the agglutination or precipitation tests between the spore or O antigens of any one of the 12 organisms and the heterologous antisera.

Table 4. *Spore and vegetative cell O antigen agglutination titres*

Organism	Antiserum to:	Agglutination titres obtained with		
		Unheated spores	Autoclaved spores	Vegetative O antigen
B. cereus M.8	Autoclaved spores	10,240	5,120	0
	O antigen	0	0	640
B. megaterium 2529	Autoclaved spores	640	640	0
	O antigen	0	0	640
B. subtilis 3610	Autoclaved spores	640	640	0
	O antigen	0	0	640
B. pumilus 2746	Autoclaved spores	80	80	0
	O antigen	0	0	320
B. licheniformis 2843	Autoclaved spores	640	320	0
	O antigen	0	0	320
B. coagulans K & P	Autoclaved spores	20,480	10,240	0
	O antigen	0	0	320
B. polymyxa 2002	Autoclaved spores	1,280	640	0
	O antigen	0	0	320
B. circulans 2925	Autoclaved spores	160	160	0
	O antigen	0	0	160
B. alvei 2198	Autoclaved spores	320	640	0
	O antigen	0	0	640
B. brevis 2934	Autoclaved spores	2,560	1,280	0
	O antigen	0	0	320
B. laterosporus 68A	Autoclaved spores	640	320	0
	O antigen	0	0	640
B. sphaericus 2546	Autoclaved spores	160	160	0
	O antigen	0	0	320

Table 5. *Spore precipitation reactions*

Organism	Results of the precipitin test with spore material
B. cereus M.8	Two strong zones near the interface
B. megaterium 2529	A single, rather weak zone
B. subtilis 3610	A single, strong zone
B. pumilus 2746	A single, strong zone
B. licheniformis 2843	A strong zone and a subsidiary zone in the antiserum column
B. coagulans K & P	A strong zone and a subsidiary zone in the antiserum column
B. polymyxa 2002	A single, strong zone
B. circulans 2925	A single, strong zone
B. alvei 2198	A strong zone and a subsidiary zone in the antiserum column
B. brevis 2934	A strong zone and a subsidiary zone in the antiserum column
B. laterosporus 68A	Two strong zones, one at the interface and one in the antiserum column
B. sphaericus 2546	A single, strong zone

Tables 1–3 indicate that the injection of H antigen stimulated the production of O antibody in addition to the homologous H antibody (cf. Davies, 1951). This however could be differentially absorbed (Tables 2 and 3).

With *B. cereus*, *B. subtilis* and *B. licheniformis* the H, O and spore antigens were quite distinct and no cross reaction could be demonstrated between them. However, with *B. alvei* the position was more complex (Table 3), since the H antiserum when absorbed with O antigen still reacted with unheated spores. The intimate relationship between H antigen and unheated spores was also evidenced by the ability of unheated spores to remove H agglutinins from the H antiserum.

Precipitinogens were associated with spores and with vegetative cells and, except in *B. alvei*, the two antigens were distinct. Those associated with the vegetative stage reacted with H antisera.

There was no evidence for a precipitin associated with O antisera.

The specificity of the different antigens

This section falls into two parts: a detailed investigation of the spore, O and H reactions of a number of strains of *B. cereus*, *B. subtilis* and *B. licheniformis* and a supporting study of the spore antigens of a further 9 species each represented by a few strains.

A spore antiserum was prepared against autoclaved spores of one strain of each of the species studied. The strains chosen were typical as judged by the criteria of Smith, Gordon & Clark, 1952.

B. cereus *M.8 antisera*

Tests were performed with 27 different strains of *B. cereus*, which included the strain *B. terminalis* (Greenberg & Halvorson, 1955) and one representative of each of the varieties *thuringiensis*, *anthracis* (an avirulent strain) and *mycoides*.

Spore antiserum. Only seven of the *B. cereus* strains (including *B. terminalis*) were agglutinated by an antiserum to autoclaved spores of *B. cereus* M.8, each agglutinating at a reasonably high titre (1,280–10,240). None of the three recognized varieties mentioned above was agglutinated. Absorption tests with the positive strains showed in all cases complete removal from the antiserum of agglutinins for spores of the homologous *B. cereus* M.8.

The antiserum gave a precipitation reaction with extracts of spores of each of the 27 strains tested. The homologous strain and 2 others gave two zones of precipitate. Both were strong reactions and occurred near the interface between the reactants.

O antiserum. The vegetative cells of only three of the strains showed agglutination with a *B. cereus* M.8 O antiserum and titres were of a lower order (160–320) than those observed with the spore agglutinins. Absorption tests with each of the positive strains gave complete absorption of all the agglutinins for the homologous organism.

The O antiserum did not give a precipitation reaction with extracts of the vegetative cells of any of the *B. cereus* strains.

H antiserum. Six of the *B. cereus* strains (including *B. terminalis*) were agglutinated by a *B. cereus* M.8 H antiserum. The titres showed wide variation (160–40,960). Agglutinin absorption tests with each of the above six positive strains showed that all were capable of absorbing the full complement of agglutinins for the homologous antigen.

Only extracts of the organisms which gave a positive H agglutination reaction gave a precipitation reaction with the H antiserum and in these one single strong zone of precipitate appeared.

B. subtilis 3610 *antisera*

The reactions of 26 different strains of *Bacillus subtilis* were investigated.

Spore antiserum. With the exception of one strain which failed to sporulate, spores of each of the 25 strains were agglutinated by spore antiserum 3610. Titres varied over a wide range (20–10,240). Absorption tests were performed with antigens of 4 strains and all absorbed the full complement of agglutinins from the antiserum.

The antiserum gave a positive precipitation reaction with extracts of spores of every one of the *B. subtilis* strains. In each case this took the form of a single strong zone of precipitate at the interface between the extract and antiserum.

O antiserum. Vegetative cells of each of the 26 strains of *B. subtilis* were agglutinated by the O antiserum to strain 3610. titres ranging from 40–1,280. Absorption tests were performed with the four strains used in the spore investigation above and all absorbed the full complement of agglutinins from the antiserum.

The O antiserum failed to give a precipitation reaction with extracts from vegetative cells of any one of the *B. subtilis* strains.

H antiserum. Ten of the 26 strains of *B. subtilis* were agglutinated by the H antiserum to strain 3610; titres ranged from 40–10,240. Absorption tests were performed with H antigen preparations of 4 of the 10 strains. In each case there was complete removal of H antibody from the 3610 antiserum.

The 10 strains giving a positive H agglutination also gave a precipitation reaction when extracts of their vegetative cells were tested against 3610 H antiserum. The reaction took the form of a single strong zone of precipitate at the extract-antiserum interface. None of the other 16 strains gave a positive reaction.

B. licheniformis 2843 *antisera*

The reactions of 18 different strains were investigated.

Spore antiserum. Spores of each of the 18 *B. licheniformis* strains agglutinated with 2843 spore antiserum, the titres ranging from 20–1,280. Absorption tests were performed with spores of 2 strains, both of which completely absorbed the agglutinins for the homologous strain.

With one exception, extracts of the spores of each of the *B. licheniformis* strains gave a precipitation reaction with the 2843 spore antiserum. In each case the reaction took the form of a single strong zone at the interface and a subsidiary weaker one within the serum column.

O antiserum. The vegetative cells of only 5 of the 18 strains agglutinated with the 2843 O antiserum. Titres ranged from 80–320. Absorption tests with 4 of the 5 strains showed that each was capable of removing the full complement of agglutinins from the 2843 antiserum.

None of the vegetative cell extracts gave a precipitation reaction with this antiserum.

H antiserum. The 2843 H antiserum agglutinated 14 of the 18 strains, titres ranging from 20–2,560. Absorption tests performed with H antigen preparations of 4 of the 14 strains showed that each of these organisms was capable of removing the full complement of agglutinins from this antiserum.

Only the strains which gave a positive H agglutination reaction showed a positive precipitation test with the H antiserum. These all took the form of a single, strong zone of precipitate at the extract-antiserum interface.

Additional species

The reactions of the spore antisera of the nine additional species tested, with those for the three species investigated in detail, have been incorporated in Table 6. With only one exception (a strain of *B. licheniformis* whose spore extract gave a negative reaction) each strain possessed a precipitinogen reacting with the spore antiserum of a typical representative of the homologous species. Spore agglutinogens showed a more variable distribution pattern, suggesting considerable subspecies specificity.

Table 6. *Reactions obtained with spore antisera tested against spores and spore extracts of strains of the homologous species*

Species and strains used to prepare antisera	No. of strains agglutinated/ no. tested, and range of titres	No. of strains giving precipitation*/ no. tested
B. cereus M.8	7/27 1,280–10,240	27/27
B. megaterium 2529	2/7 160–640	7/7
B. subtilis 3610	25/25 20–10,240	25/25
B. pumilus 2746	3/3 20–80	3/3
B. licheniformis 2843	18/18 20–1,280	17/18
B. coagulans K & P	3/3 640–20,480	3/3
B. polymyxa 2002	2/2 320–1,280	2/2
B. alvei 2198	1/2 320	2/2
B. circulans 2925	1/3 160	3/3
B. brevis 2934	1/4 2,560	4/4
B. laterosporus 68A	3/3 160–640	3/3
B. sphaericus 2546	2/3 80–160	3/3

* The types of reaction are given in Table 5.

A similar study of the distribution of vegetative O antigens indicated a great deal of subspecies specificity, suggesting that little taxonomic significance is to be attached to this antigen (cf. Davies, 1951). None of the O antisera gave precipitation reactions with vegetative cell extracts.

Species specificity of the antigens

Since in general O and H antigens appeared to be more strain specific than the spore antigens, tests for possible interspecies reactions were limited, in the main, to the latter type of antigen. Both agglutination and precipitation techniques were employed.

Spore agglutination reactions

The results of these tests are given in Table 7. Spore antisera for *B. cereus*, *B. megaterium*, *B. licheniformis*, *B. coagulans*, *B. polymyxa*, *B. circulans*, *B. brevis* and *B. laterosporus* agglutinated only spores of strains of the homologous species and showed no tendency to cross agglutinate with spores of other species; interspecies cross reactions were encountered in other species. Thus, *B. subtilis* 3610 spore antiserum agglutinated spores of each of 3 strains of *B. pumilus* tested, to titres of 1,280, 320 and 80 respectively. Spores of *B. pumilus* 2746 were able to absorb from *B. subtilis* 3610 antiserum all the spore agglutinins of the latter. The spore antiserum of *B. pumilus* 2746 gave only a low titre (20) agglutination with *B. subtilis* 3610 spores and the latter were unable to absorb completely the agglutinins for *B. pumilus*. It may be concluded that *B. pumilus* has at least two spore antigens, one of which is shared with *B. subtilis*. Further, the spore antiserum to *B. alvei* 2198 gave a trace of agglutination at a dilution of 1 in 20 with spores of *B. sphaericus* 2546 and the spore antiserum to the latter organism gave a similar trace reaction with *B. alvei* spores. Though of doubtful significance in itself, this cross reaction received confirmation from a study of spore precipitinogens (see below).

Table 7. *Interspecies cross agglutination reactions* between spore antisera and the spores against which they were produced*

Antiserum prepared from:	Cross agglutination titres obtained with											
	B. cereus M.8	*B. megaterium* 2529	*B. subtilis* 3610	*B. pumilus* 2746	*B. licheniformis* 2843	*B. coagulans* K & P	*B. polymyxa* 2002	*B. circulans* 2925	*B. alvei* 2198	*B. brevis* 2934	*B. laterosporus* 68A	*B. sphaericus* 2546
B. cereus M.8	10,240	—	—	—	—	—	—	—	—	—	—	—
B. megaterium 2529	—	640	—	—	—	—	—	—	—	—	—	—
B. subtilis 3610	—	—	640	1,280	—	—	—	—	—	—	—	—
B. pumilus 2746	—	—	†	80	—	—	—	—	—	—	—	—
B. licheniformis 2843	—	—	—	—	640	—	—	—	—	—	—	—
B. coagulans K & P	—	—	—	—	—	20,480	—	—	—	—	—	—
B. polymyxa 2002	—	—	—	—	—	—	1,280	—	—	—	—	—
B. circulans 2925	—	—	—	—	—	—	—	160	—	—	—	—
B. alvei 2198	—	—	—	—	—	—	—	—	320	—	—	†
B. brevis 2934	—	—	—	—	—	—	—	—	—	2,560	—	—
B. laterosporus 68A	—	—	—	—	—	—	—	—	—	—	640	—
B. sphaericus 2546	—	—	—	—	—	—	—	—	†	—	—	160

* A dash indicates no reaction. † A trace of agglutination at a serum dilution of 1:20.

Spore precipitation reactions

The spore precipitation tests (Table 8) indicated that a spore component of *B. circulans* was responsible for a weak and apparently nonspecific cross reaction with each of the organisms tested. The spore extract also gave a strong zone with its homologous antiserum. Further consideration of the spore precipitin tests may most conveniently be considered independently of this nonspecific reaction, which could readily be distinguished from the strong homologous reaction (and which could possibly be obviated by absorption techniques using *B. circulans* spore material against the other antisera). *B. circulans*, *B. licheniformis*, *B. polymyxa*, *B. brevis* and *B. laterosporus* gave a strong, specific reaction with their respective homologous antisera. *B. cereus* antiserum gave a strong precipitation reaction with extracts of spores of the homologous organism and a weak precipitation reaction was apparent with spore extracts of *B. megaterium*. *B. megaterium* antiserum gave a weak reaction with *B. cereus* spore extracts indistinguishable from that obtained with the homologous organism.

Minor cross reactions also occurred between *B. subtilis* and *B. pumilus*, between *B. pumilus* and *B. coagulans*, and between *B. alvei* and *B. sphaericus*, but these were readily distinguished from the stronger homologous reactions.

Table 8. *Interspecies cross precipitation reactions between spore antisera and extracts of the spores against which they were produced*

Antiserum prepared from:	Precipitation reactions* obtained with											
	B. cereus M.8	*B. megaterium* 2529	*B. subtilis* 3610	*B. pumilus* 2746	*B. licheniformis* 2843	*B. coagulans* K & P	*B. polymyxa* 2002	*B. circulans* 2925	*B. alvei* 2198	*B. brevis* 2934	*B. laterosporus* 68A	*B. sphaericus* 2546
B. cereus M.8	++	S	—	—	—	—	—	S	—	—	—	—
B. megaterium 2529	±	±	—	—	—	—	—	+S	—	—	—	—
B. subtilis 3610	—	—	+	+	—	—	—	S	—	—	—	—
B. pumilus 2746	—	—	±	+	—	+	—	±	—	—	—	—
B. licheniformis 2843	—	—	—	—	+S	—	—	±	—	—	—	—
B. coagulans K & P	—	—	—	S	—	+S	—	S	—	—	—	—
B. polymyxa 2002	—	—	—	—	—	—	+	S	—	—	—	—
B. circulans 2925	±	±	±	±	±	±	±	+	±	±	±	±
B. alvei 2198	—	—	—	—	—	—	—	S	+S	—	—	+S
B. brevis 2934	—	—	—	—	—	—	—	S	—	+S	—	—
B. laterosporus 68A	—	—	—	—	—	—	—	S	—	—	+S	—
B. sphaericus 2546	—	—	—	—	—	—	—	±	+	—	—	+

* ++, two strong precipitation zones at interface; +, strong zone at interface; ±, weak zone at interface; S, secondary zone in serum column; —, no reaction.

Vegetative O agglutination reactions

Tests based on the use of a few strains were applied to the investigation of vegetative O antigens. The results indicated numerous cross agglutination patterns involving two or, in some cases, even three species (see Table 9).

DISCUSSION

Defalle (1902) demonstrated specific spore agglutinogens in *B. mycoides*, *B. mesentericus-vulgatus*, *B. alvei* and *B. subtilis* and Mellon & Anderson (1919) concluded that there were distinctive spore and vegetative antigens in *B. subtilis*. On the other

Table 9. *Interspecies cross agglutination reactions* between O antisera and the antigens against which they were produced*

Antiserum prepared from:	Agglutination titres obtained with											
	B. cereus M.8	*B. megaterium* 2529	*B. subtilis* 3610	*B. pumilus* 2746	*B. licheniformis* 2843	*B. coagulans* K & P	*B. polymyxa* 2002	*B. circulans* 2925	*B. alvei* 2198	*B. brevis* 2934	*B. laterosporus* 68A	*B. sphaericus* 2546
B. cereus M.8	320	—	—	—	—	—	—	—	—	—	—	—
B. megaterium 2529	—	640	—	160	—	—	—	—	—	—	—	—
B. subtilis 3610	—	—	640	—	—	—	—	—	—	—	—	—
B. pumilus 2746	—	—	—	320	—	—	—	—	—	—	—	—
B. licheniformis 2843	—	—	—	—	320	—	—	—	40	—	80	—
B. coagulans K & P	—	—	—	—	—	320	—	—	—	80	320	—
B. polymyxa 2002	—	—	—	—	—	—	320	—	—	—	—	—
B. circulans 2925	—	—	—	—	—	—	—	160	320	—	—	—
B. alvei 2198	—	—	—	—	160	—	—	—	640	—	—	—
B. brevis 2934	—	—	—	—	—	20	—	—	—	320	1,280	—
B. laterosporus 68A	—	—	—	—	40	—	—	—	—	160	640	—
B. sphaericus 2546	—	—	—	—	—	—	—	—	—	—	—	320

* A dash indicates no reaction.

hand Krauskopf & McCoy (1937) failed to confirm such specific spore antigens in the *B. niger* variety of the latter species. More recently the evidence for antigens specific to the spore has become stronger (Lamanna, 1940*a*, 1942; Howie & Cruickshank, 1940; Bekker, 1944; Davies, 1951) and the present findings leave no doubt that there are antigenic differences between the two phases and that vegetative cell H, vegetative cell O and spore antigens are separate entities.

The production of vegetative cell H antibodies as a result of the injection of unheated spore suspensions can be explained in three ways: H antigen may be present in the spores themselves; the spores may be contaminated with H antigen derived from remnants of the vegetative cells; or spore germination within the animal body may result in the synthesis or unmasking of H antigen. It is not easy to distinguish between these possibilities, since quantitative as well as qualitative differences may be expected. Unheated spores of *B. alvei* absorbed H antibody from H antisera, indicating the presence of H antigen as a surface component of these spores, and this was supported by the finding that the spore extracts of *B. alvei* reacted with H antiserum. The spores of *B. alvei* retain remnants of the wall of the sporangium which could be the site of the H antigen. In this connection it may be pointed out that previous workers have implied that H antigens in the genus *Bacillus* are necessarily associated with flagella, presumably by analogy with the situation in other genera. There is no evidence to support this suggestion and, indeed, gel diffusion work leads us to believe that it is probably incorrect to regard these H antigens as flagellar in nature.

The situation with *B. cereus* and *B. subtilis* is less clear. Here unheated spores were not agglutinated by H antisera, nor would they absorb H antibody from such sera. This does not preclude their containing H antigen as a subsurface component, but the fact that formamide extracts of spores of these species did not react with H or O antisera, whereas extracts of vegetative cells reacted with the antisera resulting from injection of viable spores but not with antisera to killed spores, perhaps supports the suggestion that germination occurs within the animal body and leads to the production of antibodies specific for the vegetative phase. The striking absence of vegetative O agglutinins from any of the spore antisera might militate against this idea, but it should be borne in mind that the germination process may be arrested before active vegetative growth ensues, resulting in serological reactions about which one can but speculate. Teale & Bach (1919) demonstrated germination of spores of some aerobes following injection into animals but found little evidence of extensive growth of the vegetative forms produced. In view of their findings the possibility of germination cannot be lightly dismissed and it is worth recording that the injection of unheated spores of *B. cereus* resulted on two occasions in the death, within a few hours, of the rabbits and that vegetative bacilli were found in the blood at post-mortem. A strain of *B. cereus* apparently identical with the organism injected was isolated from the blood in pure culture.

Contamination of unheated spore suspensions by antigenic material derived from vegetative cells would not necessitate the persistence of particulate cell remnants either as free lying material or as extraneous spore membranes. Evangelinos & Wohlfeil (1939) showed that autolysis of a cell does not destroy its antigenicity. The spores of *B. subtilis* show no external membranes and the exosporium surrounding spores of *B. cereus* is not a bacillary cell wall remnant but arises as a discrete intracellular structure synthesized at sporulation (Chapman, 1956; Norris & Watson, 1960). Nevertheless the adsorption of H antigen on the surface of spores in amounts too small to cause agglutination by H antiserum but sufficient to stimulate antibody formation on injection into rabbits is at least possible, and should be considered when the results of experiments of this kind are being evaluated.

The demonstration of precipitinogens in spores (Lamanna, 1942) and in their vegetative cells (Sievers & Zetterberg, 1940; Sievers, 1942) is of great significance. In addition to confirming the independent existence of these antigens the present investigation has shown that the strain distribution of the vegetative precipitinogens parallels that of the H antigen, suggesting that the precipitinic hapten may be a component of the agglutinogen. The spore precipitinogens and agglutinogens do not run in parallel, however, and one must conclude either that there is no agglutinogen common to all strains of certain species (e.g. of *B. cereus* and *B. megaterium*) or that, if present, it is in a position or form which prevents its functioning as an antigen.

Lamanna (1940*b*) and Lamanna & Eisler (1960) have presented evidence for the existence of more than one spore agglutinogen in strains of *B. cereus* and the anthrax bacillus and in the present work this was also apparent in *B. pumilus* as shown by its relationship with *B. subtilis*. This multiplicity of antigens also applied to the precipitinogens as evidenced by the *B. subtilis–pumilus–coagulans* relationship. The presence of more than one precipitation zone obtained with gel diffusion techniques also supports this view.

One of the principal aims of the present investigation was to ascertain the extent to which the serological properties of the group might provide support for the existing scheme of classification. In the genus *Bacillus* failure to recognize the extent of strain variation led to the description of an embarrassingly large number of species and it was not until Smith, Gordon & Clark (1946, 1952) delimited the boundaries of variation within each species that material progress was made in simplifying the classification of the group. Their system, based on physiological and morphological criteria, was used as the basis for an investigation of the nutritional requirements of the genus (Knight & Proom, 1950; Proom & Knight, 1955), with results which in the main supported the soundness of the classification.

The only previous substantial serological investigation of aerobic sporeformers was that of Lamanna (1940*a*,*b*), who concluded that such an approach might have taxonomic value. His findings, however, based on agglutination reactions alone, did not present a clear picture. Later Lamanna (1942) by means of the spore precipitin technique, studied the status of *B. subtilis* and clearly demonstrated its distinction from the Ford strain (*B. licheniformis*).

In the present paper the results of the agglutination and precipitation tests have shown that, with some exceptions, both of them could be applied for taxonomic purposes. Whereas the precipitation reactions would appear to be the more species specific, the agglutination reactions also have their value and their species specificity is particularly striking in the small celled species of group I studied (e.g. *B. subtilis* and *B. licheniformis*) and in *B. polymyxa* (Davies, 1951).

The results of the present study support strongly the soundness of the classification elaborated by Smith, Gordon & Clark. It may be added in conclusion that in the course of investigating the spore precipitinogens it became possible to redesignate various organisms whose identification had obviously been in error. Confirmation of their redesignation was apparent from their morphological and physiological properties. Similarly a closer identification of several intermediate strains was also achieved.

The majority of the cultures used were obtained from the National Collection of Type Cultures, Colindale. Several strains were isolates from local soil and we are indebted to Dr. E. I. Garvie and the late Dr. J. F. Powell for several strains of *B. cereus*, to Dr. S. E. Jacobs for a culture of *B. thuringiensis*, to Mr. H. Proom for a strain of *B. cereus* var. *mycoides* and for several strains of *B. megaterium* and *B. cereus–B. megaterium* intermediates and *B. alvei–B. circulans* intermediates, to Dr. D. J. Jayne-Williams for several *B. subtilis* strains and to Dr. T. Gibson for strains of *B. megaterium* and *B. polymyxa*.

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A NOTE ON THE PRESENCE OF ANTIBACTERIAL SUBSTANCES IN TOBACCO PLANTS

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SUMMARY: The juice pressed from twelve varieties of tobacco, and materials extracted with ether from these plants, frequently displayed antibacterial activity against several species of bacteria. Some varietal difference in activity towards *Ps. tabaci* was observed, and there is a suggestion of some correlation between this and resistance to 'Wildfire'.

Antibacterial activity has been recorded from many plant species. Sherman & Hodge (1936) found it in several plant juices and Osborn (1943), who examined 2,300 plant species, found that 134 gave inhibitory extracts. Particularly active were members of the Ranunculaceae, a fact confirmed by Seegal & Holden (1945). Examinations have also been carried out by Sanders, Weatherwax & McClung (1945), Little & Grubough (1946), Carlson, Bissell & Mueller (1946), Carlson & Douglas (1948) and Carlson, Douglas & Robertson (1948). The latter tested members of the Solanaceae.

In all this work, emphasis has been placed on activity against human pathogens. Little & Grubough (1946), however, found their extracts to be also active against a number of phytopathogenic bacteria and obtained an indication that, in the case of corn, resistance to the bacterial wilt due to *Bacterium stewartii* (*Xanthomonas stewarti*) may be due to the presence of antibacterial substances, as these were found in the juice of resistant varieties.

Experiments and Results

The plant juice was obtained by macerating leaves and whole shoots from 4–5 month old plants grown in a greenhouse and filtering off the debris. The clear juice was used without sterilization. Ether extracts were prepared by adding 5 ml of pure ether to 5 g of macerated plant material, storing for 24 hr at 6° and filtering to obtain a clear extract.

The plant juices were examined by the Oxford cup method of assay and the ether extracts by the paper disc method (Vincent & Vincent, 1944; Epstein *et al.* 1944). The test organisms were *Staphylococcus aureus* and *Escherichia coli*, together with the plant pathogens *Agrobacterium tumefaciens*, *Corynebacterium fascians*, *Erwinia carotovora*, *Pseudomonas tabaci* and *Xanthomonas campestre*.

Petri dishes of 6 in. diameter were used; 60 ml of nutrient agar seeded with the test organism were poured into each, allowed to set, and the 'plates' left at room temperature for 1 hr. Sterile porcelain cylinders were then placed on the agar and filled with the plant juice. For the ether extracts, sterile 8 mm filter paper discs were dipped in the extract, allowed to drain for 5 sec and immediately placed on the agar surface. All 'plates' were incubated at 30° and examined after 24 and 48 hr.

Table 1. *The occurrence of antibacterial activity in leaf and shoot juice and in ether extracts* of leaves of twelve varieties of tobacco*

Variety	Antibacterial activity† against					
	E. coli	*A. tumefaciens*	*C. fascians*	*X. campestre*	*Ps. tabaci*	*Staph. aureus*
Bonanza	—	—	—	P (+)	+	P
Broad leaf	—	P	—	—	+(P)	P
C 7	—	P (—)	P	— (+)	+	P
Delcrest	—	—	—	+	—	—
Gold Dollar	—	P	+ (—)	P (—)	+	— (+)
Harrison's Special	—	—	—	— (P)	+	P (+)
Hickory Prior	—	—	—	—	—	—
Hicks' Special	—	P	—	P	P	—
Jamaica Wrapper	—	— (+)	+ (—)	—	+	P
Oxford 26	P (+)	— (+)	—	P (+)	+	P (+)
Special 400	—	—	—	P	+	P
White Stem Orinoco	—	—	—	— (P)	+	P

* Symbols in brackets indicate results from tests with ether extracts. These are shown only where they differed from those with expressed juice.

† Inhibition zones: +, zone of complete inhibition; P, zone of partial inhibition; —, no inhibition of any type.

The results, shown in Table 1, revealed the presence of antibacterial substances in the juices and ether extracts of the majority of the varieties tested. Inhibition of *E. coli* was restricted to the juice and ether extract of the variety Oxford 26, but some inhibitory activity against *Staph. aureus* was evident in 9 of the 12 varieties examined. Inhibition of all the plant pathogens was occasioned by one or other of the varieties, and the effects were most pronounced against *X. campestre* and, especially, *Ps. tabaci*. Inhibition of the latter organism is of special interest as a possible factor in resistance to the 'Wildfire' disease, and it will be seen that two varieties, Hickory Prior and Delcrest, yielded no inhibitory material and a third variety, Hicks' Special, gave rise only to partial inhibition. Precise information concerning the natural resistance of tobacco varieties to 'Wildfire' appears to be lacking, but Hickory Prior is known to be highly susceptible and the resistance of Hicks' Special and Delcrest very much less than that of the other varieties tested. It appears therefore that a correlation may exist between resistance to 'Wildfire' and antibacterial activity, though more information on resistance to 'Wildfire' is required before this can be confirmed.

The conditions under which the plants were grown may have affected the production of the inhibitory substances. The work was carried out over three years, one batch of plants of each variety being grown in a greenhouse during each summer, and the results in Table 1 represent the combined results from the three series of tests. Although the plants were greenhouse grown, the external conditions had a pronounced influence on the rate and type of growth. Two of the three summers were dull and cold and the leaf tissue was less fleshy and yielded less juice than in the third summer, which was sunny and warm. The sizes of the inhibition zones were likewise affected; they were smaller when the plants were grown under dull and cold conditions, and it thus appears that the conditions of growth affected the production of the antibacterial substances. It will therefore be necessary, in any further study of the connection between antibacterial activity and disease resistance, to examine plants grown under normal conditions of cultivation, when their resistance to 'Wildfire' could also be determined.

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BACTERIOLOGICAL CHANGES IN SILAGE AS AFFECTED BY LACERATION OF THE FRESH GRASS

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SUMMARY: Grass, after (a) mincing, (b) chopping (to 1–3 cm), and (c) neither treatment, was ensiled at 30° in the laboratory. The growth of Gram-negative organisms and of each of the main groups of lactic acid bacteria was fastest in the minced material and slowest in the untreated. Mincing had the special effect of permitting the multiplication of lactic acid bacteria when other silage organisms were losing viability. The pH value decreased much more rapidly and to lower levels in minced silage than it did in silage of untreated grass. Variations in response to the treatments were associated with differences in the bacterial populations on the fresh grass and with properties of the grass. Silage made in larger quantities in concrete silos from untreated or lacerated grass of high sugar content had a bacterial population dominated by lactobacilli and was of low pH irrespective of the treatment applied. Silage made at 30° in the laboratory from the same herbage showed that the effects of laceration were considerable and similar to those of mincing.

THE QUALITY of silage has frequently been found to be improved as a result o chopping or lacerating the fresh herbage. Treatments which damage the plant tissue still more severely, such as crushing, have been explored in attempts to secure further improvement. Tiemann & König (1939), Arnaudi (1940), Gneist (1944) Kappelle & Leniger (1957), Dijkstra (1958), Wieringa (1959*a*,*b*) and Gordon, Wiseman Derbyshire, Jacobson & Black (1959) have obtained evidence that the greater th disintegration of the plant tissues the more active is the lactic acid fermentation i the silage. In most of these investigations the effects of the treatments on the silag bacteria were assessed indirectly by chemical methods. Few direct bacteriologica findings are available. However, Stirling (1951) described a preliminary experimen in which the effects of mincing grass before ensilage were to enable the silage bacteri to multiply more rapidly, to reach a greater count and to form acid more actively proteolytic and Gram-negative organisms quickly disappeared. Anderson (1956 made one examination of silage of chopped and unchopped grass, which indicate that chopping had promoted the growth of acid-forming bacteria. Wieringa (1959*a*) using a nitrogen-rich grass, compared mincing with chopping. In silage incubate at either 20° or 35° bacteria capable of growth on acetate agar multiplied mor rapidly and reached denser populations in the minced material. This was associate with lower pH levels and smaller concentrations of butyric acid in the minced silage

The object of the present work was to examine the effects on the bacteria populations in silage of liberating cell sap by mechanical treatment of the fres herbage.

METHODS

Laboratory-made silage. Perennial ryegrass, handled as aseptically as possible, was incubated, with exclusion of oxygen, at 30° in 50 g quantities. The grass was ensiled in three states: (a) untreated, (b) chopped into 1-3 cm lengths and (c) after passing through a sterilized household mincing machine. The specimens of grass used for ensilage have been described previously (Gibson. Stirling, Keddie & Rosenberger, 1958).

Silage made in small concrete silos. Outdoor cylindrical silos of two sizes were used. The larger, 6 ft high and 4 ft in diameter, were filled with 860 kg of herbage; the smaller, 3 ft high and 3 ft in diameter, with 250 kg. The herbage was thoroughly tramped during filling, then covered with waterproofed paper and 300 kg or 200 kg of soil, for the larger and smaller silos respectively. Rain was excluded by additional covers.

One-year ryegrass in the preflowering stage and containing a small proportion of clover was ensiled in May. Its average dry matter content was 21%, and the average crude protein content was 12·8% of the dry weight. Soluble carbohydrates, excluding oligosaccharides, formed 24·8% of the dry weight (Wylam, 1953). The herbage was ensiled without mechanical treatment and also after being passed through a machine having a lacerating action. Molasses, when used, was mixed with an equal volume of water and the diluted material was applied at the rate of 4 gal/1,000 kg.

Examination of grass and silage. The methods used have been described previously by Gibson *et al.* (1958) and they are only briefly outlined here. The first step was to macerate 50 g of grass or silage in sterile water. The media employed were:—

(1) Glucose yeast agar. Colony counts of organisms other than clostridia were estimated on this medium after incubation for 5 days at 30°.

(2) Acetate agar, pH 5·4. Double layer plates were incubated for 5 days at 30°. A sample of colonies was examined in order to estimate counts of lactobacilli because leuconostocs and pediococci also grow on this medium.

(3) Lactate agar containing glutamic acid and crystal violet. Counts of Gram-negative bacteria were estimated after incubation for 3 days at 30°.

(4) Lactate acetate semisolid agar was used for lactate fermenting anaerobes.

(5) Peptone gelatin medium was employed for proteolytic anaerobes.

Tubes of media (4) and (5) were inoculated in triplicate from dilutions of macerate and incubated at 30° or 37°. Cultures in (4) were examined for gas formation and increase in pH value; those in (5) were tested for liquefaction and production of NH_3 and H_2S. From the results an estimate was made of the most probable number of each type of anaerobe.

RESULTS

Changes in laboratory-made silage

Examination of the fresh grass yielded colony counts in the range of $2{\cdot}6\text{-}220 \times 10^6$/g dry weight. An overwhelming majority of the organisms were strict aerobes which perished soon after ensilage of the grass. Bacteria capable of growing anaerobically in silage were minorities of which Gram-negative rods were the largest.

The development of bacterial populations in the silage is shown in Fig. 1 by changes in total viable count. The totals include the clostridia. In experiment 8 some of the results are incomplete because counts of clostridia were not secured, and th silage of chopped grass incubated for 180 days was rejected because anaerobi conditions were not maintained in it.

In all the silage there was a phase of active bacterial multiplication, lasting i most cases about 2 days, followed by a phase of decreasing viable count. Fig.

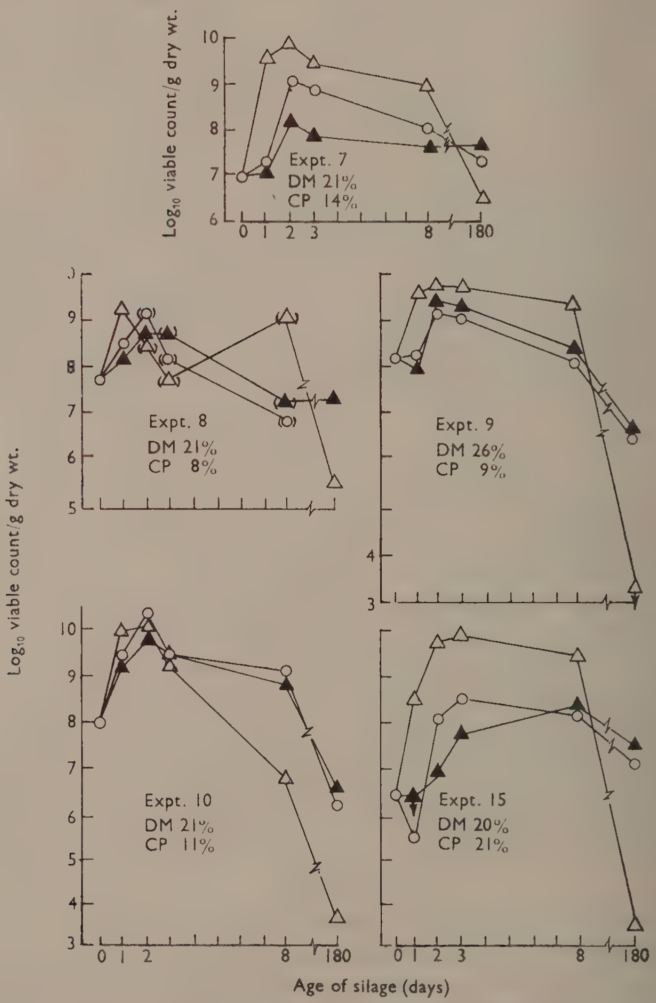

Fig. 1. The counts of total viable bacteria at different times in silage made from untreated grass (full triangles), chopped grass (circles) and minced grass (open triangles). DM, dry matter in fresh grass; CP, crude protein, % of dry weight. Symbols in brackets indicate incomplete counts.

shows that the release of plant juice by a mechanical treatment of the grass accelerated bacterial growth and increased the maximum density of viable organisms. The time at which growth was initiated is not brought out clearly by the graphs because in the first stages the bulk of the grass organisms were disappearing while the silage bacteria were multiplying. Counts on selective media and the identification of isolates showed that the bacteria which developed must have started to multiply with little delay after the silos were closed.

Fractions of the bacterial populations. The viable organisms were separated into four fractions as follows:—

(1) *Gram-negative organisms.* In most of the silage the dominant Gram-negative bacteria had characters linking them with the klebsiella group although many strains showed an atypical action on lactose, and some failed to form gas from glucose. *Bacterium herbicola* appeared less frequently and *Escherichia coli* only rarely.

(2) *Clostridia.* No obligate anaerobes other than clostridia were detected. The lactate fermenters comprised *Cl. butyricum*, an organism resembling *Cl. paraputrificum*, and, less commonly, *Cl. tyrobutyricum.* The actively proteolytic species were *Cl. bifermentans* and *Cl. sporogenes.* Species possessing neither property were detected occasionally. They were *Cl. sphenoides* and *Cl. welchii* (*Cl. perfringens*). The counts of clostridia were obtained by combining the counts of the different anaerobes.

(3) *Lactobacilli.* The results for this group were derived from the proportionate representation of the organisms on the acetate agar plates. Cultures that were examined in detail (Keddie, 1959) were found to be typical or variant forms of *L. plantarum*, *L. acidophilus*, *L. fermenti* and occasionally *L. casei.* Other isolates could not be identified with known species.

(4) *Remainder.* This designation is used for that part of the count on glucose yeast agar which could not be accounted for by Gram-negative bacteria and lactobacilli. Included in the fraction are members of the genera *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Bacillus*, organisms for which selective culture was not used. The figures for the Remainder cannot be accepted without reserve, partly because they were derived indirectly from other counts and partly because a population of bacteria, more especially after exposure to unfavourable conditions, may not give the same colony count on two different media.

Development of the bacterial groups. The counts of each fraction are set out in Table 1. They reveal considerable variation among the experiments. At the same time the responses of the organisms to the liberation of plant cell sap had a remarkable uniformity. The figures for the early stages of growth, together with supplementary information secured by identifying the organisms, show that the development of Gram-negative rods and of each of the main groups of lactic acid bacteria was most rapid in the minced material and slowest in the untreated. Further, the counts attained by all these groups were usually highest when the grass had been minced and lowest when untreated. The growth of *Clostridium* and *Bacillus* strains was more erratic. Organisms of these genera showed little or no development where lactic acid bacteria multiplied most vigorously. This occurred especially in the minced silage. Some sort of antagonism is thereby suggested. As a mechanism, acid production might be suspected, but the pH data for the early stages (Fig. 2) do not afford clear

Table 1. *The development and death of different groups of bacteria in silage made in the laboratory at 30°*

Experiment no.	Bacterial group	Viable counts (10^6/g dry wt. of original grass) in silage made from														
		Untreated grass, after (days)					Chopped grass, after (days)					Minced grass, after (da			
		1	2	3	8	180	1	2	3	8	180	1	2	3	8
7	Gram-negative	20	130	28	14	<0·1	17	1600	380	18	<0·1	2100	66	<0·1	<0·1
	Clostridia	0·4	2·5	14	12	45	<0·1	4·0	16	20	24	1·3	2·4	25	<0·1
	Lactobacilli	<0·1	*	<0·1	<0·1	<0·1	<0·1	3·0	*	*	*	*	*	*	880
	Remainder	<0·1	77	36	30	0·4	4·7	9·0	440	130	2·5	2400	7300	2900	190
8	Gram-negative	150	540	450	<0·1	<0·1	280	350	150	<0·1	†	1000	160	<0·1	<0·1
	Clostridia	<0·1	1·3	†	†	24	2·4	†	†	†	†	0·5	†	†	†
	Lactobacilli	<0·1	0·1	0·1	<0·1	<0·1	<0·1	<0·1	<0·1	<0·1	†	<0·1	<0·1	*	540
	Remainder	53	71	32	0·8	<0·1	<0·1	700	33	5·5	†	840	120	75	2200
9	Gram-negative	90	720	1800	170	<0·1	150	1500	1400	140	<0·1	1800	290	<0·1	<0·1
	Clostridia	<0·1	<0·1	0·4	0·4	1·4	<0·1	1·3	0·2	0·1	<0·1	<0·1	<0·1	<0·1	<0·1
	Lactobacilli	0·8	1·1	16	24	2·6	1·1	29	*	69	2·1	1200	2200	4200	2000
	Remainder	13	2100	<0·1	26	0·5	1·0	630	22	<0·1	0·3	900	2600	700	<0·1
10	Gram-negative	2000	2400	98	6·6	0·8	2400	2600	760	1·4	1·7	1200	7·4	<0·1	<0·1
	Clostridia	<0·1	0·9	0·6	0·2	0·1	2·5	0·5	0·1	<0·1	<0·1	<0·1	<0·1	<0·1	<0·1
	Lactobacilli	280	280	1400	1200	0·1	2800	2100	1500	1200	<0·1	6200	5200	1400	4·9
	Remainder	300	1600	870	<0·1	1·3	<0·1	18000	500	100	0·4	1700	15000	580	0·6
15	Gram-negative	<2·8	<0·3	14	9·1	<0·1	<0·3	19	78	1·0	<0·1	280	51	<0·1	<0·1
	Clostridia	<0·1	<0·1	<0·1	11	10	<0·1	14	5·6	16	6·7	5·7	<0·1	0·1	<0·1
	Lactobacilli	<0·1	<0·1	<0·1	*	<0·1	0·1	4·4	13	8·5	1·5	14	4000	7000	2500
	Remainder	<0·1	9·8	32	106	15	0·3	64	170	73	1·5	<0·1	1100	<0·1	<0·1

* No confirmation that lactobacilli were represented on acetate agar plates. † Data incomplete.

evidence on the point. The complexity of this question is illustrated by the multiplication of clostridia—they were lactate fermenters—at relatively low pH levels in the minced silage of experiment 7.

In the silage made from chopped grass no bacterial group showed, in the period up to 8 days, much ability to multiply after the others had reached the phase of decline. This was evident even when the pH value (Fig. 2) could not be regarded as unsuitable for growth. These observations are consistent with the view that growth was arrested chiefly by exhaustion of the food supply. The silage of untreated grass was similar, although clostridia and lactobacilli did show increases in some instances after the total count had passed its peak. Such late growth may perhaps be attributed to slowness in the exudation of cell sap from the plant tissues. The minced silage was quite different. During the rapid multiplication which occurred in it, lactic acid bacteria (if they were present) soon overtook the other organisms and continued to multiply when the others were losing viability. Gram-negative rods, the organisms which would have a numerical advantage at the start, showed most distinctly the early arrest of growth. The rapid and sustained growth of lactic acid bacteria was associated with steep falls in the pH curves (Fig. 2). It may be inferred that in minced silage acid became a selective factor before exhaustion of nutrients stopped the growth of the lactic acid bacteria. In experiment 10, where the pH value reached 4·7 by 24 hr, the inhibition of Gram-negative organisms occurred especially early. It would thus appear that the essential effect of liberating plant juice is to promote decisively the growth of lactic acid bacteria relative to that of other organisms. The experiments did not show clearly if there were differences among the several groups of lactic acid formers in their response to cell sap during the multiplication phase.

Variations in the results. Most of the variations and irregularities shown in Fig. 1 and Table 1 appear to be attributable either to fluctuations in the counts on grass of particular silage bacteria or to differences in the properties of the grass specimens.

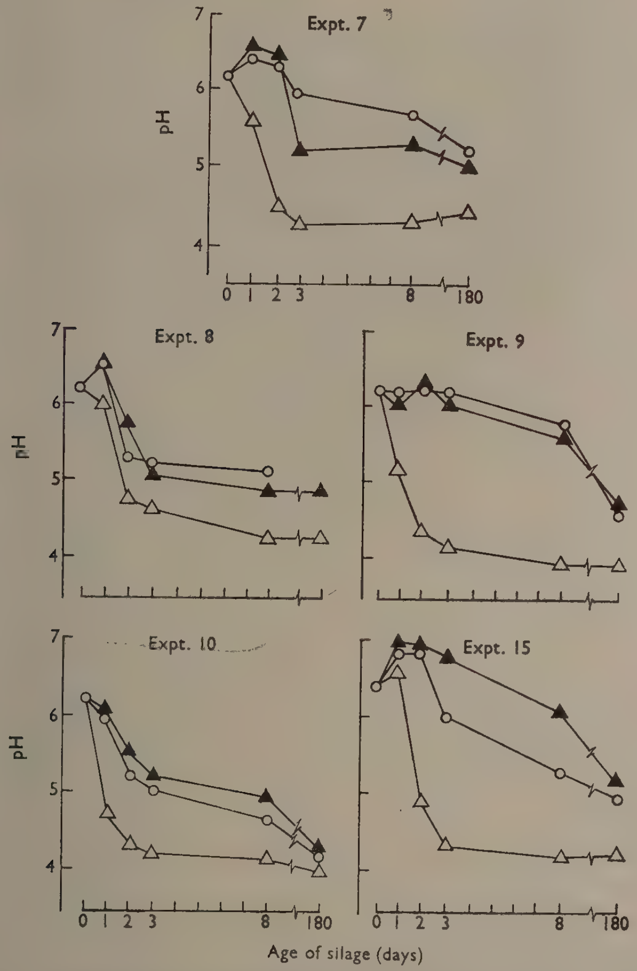

Fig. 2. The pH values of silage macerates in relation to the age of silage made from untreated grass (full triangles), chopped grass (circles) and minced grass (open triangles).

Considerable variation was associated with the distribution of lactic acid bacteria on the grass. The clearest evidence on this point concerns the genera *Lactobacillus* and *Leuconostoc*, organisms which could be detected by means of acetate agar. The grass used in experiment 10 gave an unusually high count of lactobacilli (5,000/g dry wt.), and their growth in all three types of silage was more abundant than it was in the other experiments. In experiments 9 and 15 the initial lactobacillus counts were 22 and 29/g dry wt., respectively, while in experiments 7 and 8 no colonies developed in acetate agar inoculated with 1 ml of undiluted grass macerate. In the silage of experiment 7 leuconostocs and possibly streptococci multiplied more extensively than lactobacilli, even in the minced material which in other cases permitted lactobacilli to develop freely. This suggests that lactobacilli were not sufficiently numerous at the start to enable them to compete effectively with the cocci. On the grass used in experiment 8 the seeding with lactic acid bacteria seems to have been so light that they did not occur in all the 50 g quantities ensiled. It is reasonably certain that lactobacilli were absent from most. In the silage made from this grass the bacterial populations were dominated, except in some of the minced material, by Gram-negative rods and clostridia.

The rates at which the bacteria of the several groups lost their viability (Table 1) determined the shapes of the curves in Fig. 1. Gram-negative organisms were generally the first to disappear, and where they represented a large proportion of the population the total viable counts showed steep falls before 8 days. The occurrence of Gram-negative rods at 6 months in experiment 10 appears to be unusual. The organisms were of an exceptional type which has not been detected in other silage. Lactobacilli commonly remained viable longer than the other groups. A reason for their rapid disappearance in the minced silage of experiment 10 has not been found. The pH value in this case was 4·2, yet in other silage having lower values lactobacilli retained viability much more persistently.

The five specimens of grass differed in their capacity to support bacterial growth when they were ensiled in the untreated state. This is apparent after allowing for differences in the bacterial populations on the freshly harvested grass. The magnitude of the response to mincing should show how efficiently the untreated herbage supplied the moisture and nutriment necessary for bacterial growth. Fig. 1 and Table 1 show that in experiment 10 the differences between the treatments were less than in the other experiments. The differences were large in experiment 7 and still larger in experiment 15. Another feature of the grass used in experiment 15 was that, judging by the slowness of the growth in the minced material, its cell sap may have been somewhat inhibitory.

Acid formation. For each specimen of grass the changes in pH value may be taken to indicate the relative rates of acid production in the three types of silage. The most active acid formation invariably occurred in the minced silage (Fig. 2). Chopping had a much smaller effect. In experiments 9, 10 and 15 the pH curves up to 8 days were related to the development of lactic acid bacteria, most closely to that of lactobacilli, the group which in these experiments dominated the bacterial populations of the minced silage. In experiment 7, in which lactobacilli were not numerous, the relationship was less distinct and in experiment 8, in which lactic acid bacteria of all groups were scarce except at the 8-day examination of the minced silage, the decreases in pH value did not exhibit a distinct association with the growth of any particular organism. There is no reason to doubt that the acidification of silage is attributable to acids formed by bacteria, yet these experiments show that the identification of the causal organisms may not always be simple. One complication could be the capacity of lactic acid bacteria to continue to form acid when the population is in the phase of decreasing viable count. This appears to have occurred in the present experiments.

In silage made from untreated grass the accessibility of sugars to the organisms might be a variable, and it might be one of the factors which limit the rate of acid formation. In each of the experiments the minced silage may be taken as a standard by which to assess the availability of sugars in the silage made from the untreated grass. The curves in Fig. 2 show that the difference between minced and untreated grass in rate of acid formation varied considerably. The bacteriological data do not convincingly explain all the variations. This implies that there were differences in the rate at which sugars were liberated from the plant tissues. In the silage of untreated grass in experiments 9 and 15 much of the decrease in pH value occurred after the eighth day, indicating that sugars were slow to reach the sites of bacterial growth.

None of the grass used in these experiments can have been markedly deficient in sugars. There was a tendency for the pH curves to rise in the case of the minced silage in experiments 7 and 15, suggesting that in these instances barely sufficient sugar was available to provide the acid required to balance the neutralizing changes. In the method here adopted the minced silage was very wet and the control over undesirable changes may have been weaker than if drainage had occurred.

Changes in silage made in concrete silos and in the laboratory

During the filling of the concrete silos portions of the variously treated herbage were put up for incubation in the laboratory at 30°. The laboratory silage was examined after 2, 3 and 8 days, and all the silage after about 3 months when the concrete silos were opened. The results are shown in Table 2.

The laboratory work demonstrated that the herbage was only lightly seeded with lactobacilli; leuconostocs alone were detected on the acetate agar plates in the first 3 days. Laceration of the grass had effects comparable to those already described for mincing. It evidently permitted a late growth of lactobacilli. The addition of molasses to herbage which was otherwise untreated had less influence than laceration alone, while the addition of molasses to lacerated herbage appeared to assist Gram-negative organisms and clostridia and to give no benefit to leuconostocs and lactobacilli.

In the concrete silos, judging by the results obtained when they were opened, the events were markedly different. The counts of lactobacilli were large; there were relatively few clostridia; the pH values were low. A detailed survey of the contents of each silo disclosed that all the pH figures, including those for the bottom and top layers, fell in the narrow range of 3·8–4·2. All the silage was excellently preserved. The concrete silos thus failed to provide evidence on the effects of laceration.

The chief condition in which the concrete silos differed from those in the laboratory was presumably their lower temperature. The temperatures in this experiment are unknown, but from other work on the same silos it may be assumed that they rarely if ever reached 20° and that for most of the time they were in the range 11–16°.

Table 2. *The viable counts of different groups of bacteria and the changes in pH values in silage made from the same crop in the laboratory at 30° and in concrete silos*

Treatment of herbage	Bacterial group	Viable counts (10^6/g dry wt.) and pH values† for					
		Laboratory silage after (days)				Silage‡ in concrete silos	
		2	3	8	99	4-ft silos	3-ft silos
None	Gram-negative	4800	930	48	<0·1	<0·1	Not done
	Clostridia	27 (5·5)	6·6 (4·9)	9·1 (5·2)	26 (4·6)	1·4 (3·9)	
	Lactobacilli	*	*	30	*	1700	
	Remainder	11	3800	180	0·9	<0·1	
Lacerated	Gram-negative	47	0·8	<0·1	<0·1	1·8	<0·1
	Clostridia	<0·1 (4·6)	<0·1 (4·4)	0·5 (4·3)	1·3 (4·0)	<0·1 (3·9)	<0·1 (3·9)
	Lactobacilli	*	*	270	1200	3700	1300
	Remainder	7800	5100	270	<0·1	<0·1	<0·1
Molasses (2 gal/1000kg)	Gram-negative	1200	410	0·3	<0·1	<0·1	<0·1
	Clostridia	0·1 (5·2)	1·2 (4·9)	7·9 (4·9)	13 (4·1)	0·5 (3·9)	2·6 (4·2)
	Lactobacilli	*	*	220	0·1	1400	1200
	Remainder	4200	2500	270	<0·1	<0·1	<0·1
Lacerated plus molasses	Gram-negative	690	170	<0·1	<0·1	<0·1	<0·1
	Clostridia	0·5 (4·7)	0·1 (4·5)	2·5 (4·4)	13 (4·1)	<0·1 (3·9)	<0·1 (4·0)
	Lactobacilli	*	*	*	63	110	630
	Remainder	4100	3200	780	<0·1	<0·1	<0·1

* No confirmation that lactobacilli were represented on the acetate agar plates. † pH values are given in brackets.
‡ Data obtained after 83–93 days.

Such temperatures could be expected to restrict the growth of clostridia much more effectively than that of several species of the lactic acid bacteria. They would also favour the survival of lactobacilli. In other work lactobacilli were found to retain viability in silage much longer at 10° or 20° than at 30° (Keddie, 1954).

The grass used in this trial had two features that could be regarded as important in making silage: it was rich in sugars (see Methods) and it carried few lactobacilli. In the untreated state it did not preserve well at 30°; either laceration or a much lower temperature permitted sufficient acid formation to ensure stability.

Discussion

These experiments show that the development of bacteria in silage is markedly assisted by treating the fresh herbage mechanically so that its juices are released. The outstanding bacteriological effect of liberating the plant sap was to increase the growth of lactic acid bacteria during the multiplication phase which followed immediately after filling the silos. If sufficient juice was available these organisms were still able to multiply when pH values were becoming low and other bacteria had reached the phase of decline.

It may be noted that the grass which has been described as untreated and used as the reference material in the laboratory work would not be wholly undamaged. It was harvested when relatively short, thus providing many cut surfaces, and during packing of the tubes it probably suffered substantial damage. However, the experiments give a clear indication that when herbage is ensiled in a relatively undamaged condition there will be many instances in which the nutritive conditions on the plant surfaces do not allow the lactic acid bacteria to compete effectively with other organisms during the phase of general multiplication. In such circumstances acid production would be slow. Most of the biochemical changes in silage, whether they are due to plant enzymes or to bacteria, are known to diminish as the pH value decreases (Virtanen, 1937). An early and active lactic acid fermentation should, therefore, have a greater preservative effect than one which consumes the same amount of sugar but more slowly. It seems probable that a mechanical disintegration of herbage will invariably promote preservation. Nevertheless, no treatment of this sort could be expected to ensure the stability of all silage. A weakness in any of the other factors concerned in the preservation of silage, such as the content of sugars or of moisture, could undoubtedly annul the advantage of an early production of lactic acid. Instances of silage failing to preserve although made from severely crushed grass are given by Crasemann & Heinzl (1949), de Man (1953) and Wieringa (1959*a*). Relevant to this question is the demonstration by Ruschmann, Bartram & Rinckleben (1942) and Ruschmann & Bartram (1943) that whereas the press juice of nitrogen-rich herbage fails to preserve when kept anaerobic, the addition of sufficient sugar enables lactic acid bacteria to stabilize the juice.

Apart from the release of sap, mechanical treatments have other effects which are known to assist preservation. They allow of good mixing with supplements and they facilitate consolidation. Unsupplemented herbage may also benefit from mixing. De Man (1952) suggested that since the stems of grass are richer in carbohydrate and poorer in protein than the leaves, an important effect of a crushing process is to secure uniformity. Another possible effect of mechanical processes is to distribute the lactic acid bacteria over the plant surfaces.

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FURTHER STUDIES ON THE DISINFECTION OF COWS' TEATS PRIOR TO MILKING

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SUMMARY: A comparison has been made of the disinfecting efficiency on cows' teats of a hypochlorite and a quaternary ammonium disinfectant. Little difference in efficiency was found between the disinfectants when used at 200 p/m. Hypochlorite was slightly more effective in eliminating coli-aerogenes bacteria, but there was no evidence of greater survival of other Gram-negative organisms with the quaternary disinfectant. Micrococci were the main survivors and, in summer, coagulase positive staphylococci formed a considerable proportion of these.

Efficient washing and disinfection of the udder and teats of the dairy cow prior to milking form an integral part of clean milk production. In this country the common practice is to wash with water, then to dry with a cloth wrung out in disinfectant water. Approved hypochlorite disinfectants have commonly been used in udder wash waters, but in recent years quaternary ammonium compounds (QACs) have been increasingly employed. It has been claimed that the latter possess several advantages over hypochlorites for this purpose, e.g. they do not irritate the skin and, being less affected by the presence of organic matter, their disinfecting action is prolonged.

In a previous investigation McKenzie & Rosborough (1958) compared the efficiencies of hypochlorite and a QAC when both were used at a strength of 200 p/m. Little difference was found, although the QAC gave slightly better results during the summer months. With both disinfectants the surviving bacteria were mostly micrococci and no difference could be found in the survival of coli-aerogenes bacteria. This investigation was open to the criticisms that the strength of the hypochlorite disinfectant was very much lower than that recommended by manufacturers and other investigators and that no control group of cows was employed. It seemed desirable, therefore, to carry out a fuller investigation: the results of this are presented below.

Materials and Methods

The proprietary hypochlorite used was one of the brands approved by the Ministry of Agriculture, Fisheries and Food and contained approximately 10% available chlorine. The hypochlorite was tested in two concentrations, giving approximately 200 and 600 p/m available chlorine. The QAC employed was a proprietary brand containing benzalkonium chloride and understood to contain 5% of active agent. The QAC wash was prepared according to the manufacturer's instructions, the concentration of active agent in the wash water being approximately 200 p/m. Clean warm water was used to wash a control group of cows.

Two pails of wash water containing the appropriate strength of disinfectant were prepared for each of four groups of six cows, a separate udder cloth being used in each pail. The udder and teats were washed thoroughly with the cloth from the first pail and were then dried with the cloth from the second pail. The teats were swabbed immediately after drying. The swab consisted of 4–6 in. of $1\frac{3}{4}$ in. wide medical gauze bound to a 6 in. wire fitted to a rubber stopper. The rubber stopper was inserted in a $6\times\frac{5}{8}$ in. test tube containing approximately 10 ml of swab solution. The swab solution for the teats washed with hypochlorite was quarter-strength Ringer's solution to which 0·1% of $Na_2S_2O_3.5H_2O$ had been added to remove residual chlorine. For the teats washed with the QAC the swab solution consisted of 2% (w/v) of lecithin and 3% (w/v) of Lubrol W. The swabbing was done by drawing the sterile swab twice over the external surface of each teat. one swab being used for the four teats of each cow. The swab was then returned to the test tube containing neutralizing solution.

Examination of the swab solutions was made within one hour of swabbing, the following tests being made: 1 ml quantities of 10^{-1}–10^{-3} dilutions of the solutions were plated on Yeastrel milk agar; presumptive coli-aerogenes tests were made by inoculating 1 ml quantities of the solution and 1 ml of the 10^{-1} dilution into MacConkey's bile salt broth; a coli-aerogenes count was also estimated by plating 1 ml quantities of the solution on violet red-bile agar; a test for milk souring organisms was carried out by inoculating 1 ml of the swab solution into litmus milk. The milk agar plates, MacConkey's broths and litmus milks were incubated at 30° for 72 hr and the violet red-bile plates at 37° for 24 hr.

The proportion of the surviving types of bacteria was estimated by picking a statistical number of colonies from the milk agar plates using the method of Harrison (1938). The colonies were transferred to glucose broth and incubated for 2–3 days at 30°. Subcultures were made in litmus milk and on glucose agar slopes. After a further 24–48 hr incubation at 30° the litmus milk reaction was recorded, together with the results of Gram stains and catalase tests. Towards the end of the series of tests the glucose broth cultures were retained until examination of the Gram stains was complete. Any suspected staphylococci were further examined by plating the glucose broth culture on phenolphthalein diphosphate agar following the technique of Barber & Kuper (1951).

Inoculations were made from the positive bile salt broths into further tubes of bile salt broth which were incubated at 44° to detect the presence of organisms possibly of faecal origin.

Tests for the chlorine content in the pails of hypochlorite disinfectant were made by adding 0·5 ml of 10% potassium iodide to 10 ml of wash water and estimating the chlorine strength by means of a BDH comparator disc for values between 5·0 and 250 p/m of chlorine.

RESULTS

The trials were carried out between August 1958 and July 1959 and the results have been divided into a summer period (the first and last parts of the trials) and a winter period (the middle part of the trials). During the summer period the cows were at pasture but in the winter period they were housed all day. In the tabulation of the results the summer, winter and combined periods of the experiment have been set out separately.

Under the normal conditions of milk production which applied in these trials it was obvious that complete sterility of the skin of the teats would seldom be obtained. From examination of the range of counts it was considered that a satisfactory condition was attained when the count was $<10^3$/ml of swab solution.

Table 1. *The distribution of colony count/ml from swab solutions of teats washed with hypochlorite, QAC or water*

Period	Disinfectant and concn. (p/m)	Percentage of uncountable swabs	Percentage of countable swabs with counts/ml in the ranges			
			$<10^3$	10^3–5×10^3	Over 5×10^3–25×10^3	$>25\times10^3$
Summer*	Hypochlorite (200)	13·3	42·3	40·4	15·4	1·9
	Hypochlorite (600)	5·0	45·6	28·1	14·0	12·3
	QAC (200)	15·0	39·2	31·4	25·5	3·9
	None (water only)	8·3	36·4	25·5	21·8	16·3
Winter†	Hypochlorite (200)	17·6	14·3	14·3	42·8	28·6
	Hypochlorite (600)	8·8	22·6	54·9	19·3	3·2
	QAC (200)	41·2	20·0	45·0	20·0	15·0
	None (water only)	0·0	0·0	20·6	44·1	35·3
Combined	Hypochlorite (200)	14·9	32·5	31·25	25·0	11·25
	Hypochlorite (600)	6·4	37·5	37·5	15·9	9·1
	QAC (200)	24·4	33·8	35·2	23·95	7·05
	None (water only)	5·3	22·5	23·6	30·3	23·6

* 60 swabs examined for each treatment. † 34 swabs examined for each treatment.

In Table 1 the results of the plate count tests have been assembled in four ranges: $<10^3$, 10^3–5×10^3, over 5×10^3–25×10^3 and $>25\times10^3$/ml of swab solution. The results are given as percentages of the numbers of countable swabs. In summer little difference was found between the various treatments; the highest proportion of counts in all four groups fell in the $<10^3$/ml range. The counts were higher in winter when the cows were housed all day and more bedding was used in the cowshed. In this period the 200 p/m hypochlorite group and the control group had the greatest proportion of swabs with counts in the range of 5×10^3–25×10^3/ml but with the 600 p/m hypochlorite and QAC solutions the greatest proportion of swabs occurred in the 10^3–5×10^3/ml range. In winter a high proportion (41·2%) of the plates from the QAC swabs showed spreading growths.

The results of some of the coli-aerogenes tests are shown in Table 2. Of the 376 swabs examined, 81 gave a positive result in bile salt broth: but of these only 34 showed coli-aerogenes bacteria on violet red-bile agar. A further 13 swabs, which were negative in bile salt broth, gave a positive growth on violet red-bile. As expected the greatest number of positive coli-aerogenes swabs occurred in the water controls. The incidence of these organisms was low on the teats treated with hypochlorite; a higher incidence was found in the QAC swabs. The incidence of 44° positives tended to be slightly higher in the summer series of swabs. Of the 352 swabs plated on violet red-bile agar only 47 showed growth; the distribution of range of counts of these samples was as follows: 37 had counts of less than 10/ml; 6 had counts of 10–100 ml and 4 had counts in excess of 100/ml. It would appear, therefore, that only small numbers of coli-aerogenes bacteria survived.

Table 2. *The incidence of coli-aerogenes bacteria in swab solutions of teats washed with hypochlorite, QAC or water*

Period	Disinfectant and concn. (p/m)	No. of bile salt broth tubes positive at	
		30°	44°
Summer*	Hypohclorite (200)	5	3
	Hypochlorite (600)	8	2
	QAC (200)	15	6
	None (water only)	20	6
Winter†	Hypochlorite (200)	6	2
	Hypochlorite (600)	3	0
	QAC (200)	8	2
	None (water only)	16	5
Combined	Hypochlorite (200)	11	5
	Hypochlorite (600)	11	2
	QAC (200)	23	8
	None (water only)	36	11

* 60 swabs examined for each treatment. † 34 swabs examined for each treatment.

Table 3. *The compositions of the bacterial floras from swab solutions of teats washed with hypochlorite, QAC or water*

Period	Disinfectant and concn. (p/m)	No. of cultures	Composition of flora (%)				
			Strepto-cocci	Micro-cocci	Gram-negative rods	Gram-positive rods	Miscel-laneous
Summer	Hypochlorite (200)	460	2·6	75·5	5·6	15·0	1·3
	Hypochlorite (600)	468	0·6	82·2	4·1	12·6	0·4
	QAC (200)	477	0·6	75·8	3·3	17·4	2·9
	None (water only)	482	1·0	81·8	7·9	9·1	0·2
Winter	Hypochlorite (200)	301	0·3	65·5	1·0	32·2	1·0
	Hypochlorite (600)	311	0·6	62·5	1·9	35·0	0·0
	QAC (200)	255	1·2	48·6	1·6	47·0	1·6
	None (water only)	319	1·6	71·8	1·3	24·7	0·6
Combined	Hypochlorite (200)	761	1·7	71·5	3·8	21·8	1·2
	Hypochlorite (600)	779	0·6	74·3	3·2	21·6	0·3
	QAC (200)	732	0·8	66·4	2·7	27·6	2·5
	None (water only)	801	1·25	77·8	5·2	15·35	0·4

During the course of the experiment 3,073 colonies were picked from the Yeastrel milk agar plates; 761 from the 200 p/m hypochlorite plates, 779 from the 600 p/m hypochlorite plates, 732 from the QAC plates and 801 from the control plates. The percentage distribution of the main types of organisms isolated is shown in Table 3. No marked differences in the residual flora with the various udder washes were observed. Of the 792 cultures of cocci isolated between March and July 1959, 20·3% were found to be coagulase positive staphylococci. In March this percentage was only 4·7 but the proportion rose steadily as the summer progressed and reached 39·4 for the last test in mid-July 1959.

The tests for milk souring organisms did not yield any information additional to that obtained from the total count and coli-aerogenes tests or from the examinations of the residual flora. Accordingly the results of these tests have not been presented.

Tests for the persistence of chlorine in the pails of hypochlorite wash water showed that the concentration decreased slightly after each group of six cows had been washed.

DISCUSSION

No generally accepted procedure is available for determining the efficiency of disinfectants as skin germicides. Nungester & Kempf (1942) devised an 'infection-prevention' test which entailed the direct application of disinfectant to contaminated skin. The effectiveness of the disinfectant was measured by its ability to prevent infection of the animal by organisms present on the skin and capable of causing infection if the disinfectant had not been applied. Gardner (1946, 1948) studied the disinfecting efficiency of various compounds on unwashed skin. A standard, measured area of skin (24 mm diameter) was moistened with a suspension of a normal skin commensal (*Staphylococcus albus*). The area was allowed to dry, then moistened with the disinfectant solution, which was allowed to act for different times. The area was then swabbed and nutrient agar plates prepared from the swabs. Neither of these techniques was suitable for the present investigation. Moore (1957) studied the effect of bactericides in udder wash waters by determining, after different udder treatments, the bacterial count of the freshly drawn milk or the numbers of bacteria surviving on the teat surface. The latter was measured after treatment by immersing a teat to the base for 15–20 sec in 100 ml of sterile water, and plating the resulting suspension.

In our experiments the principal object was to compare the efficiencies of the disinfectants under farm conditions. Accordingly the teats were swabbed at milking time, and as soon as possible after the udder had been washed and dried, to avoid interference with the milking routine. Thus a period of not more than 2–3 min elapsed between drying and swabbing. For a period of 4 weeks swabbings were made both before and after machine milking, but the results showed little difference in either the numbers or types of organisms and this comparison was therefore not continued. The cows used were chosen at random from a herd of sixty and varied from week to week. The hair on the udders was clipped after the animals were housed and was kept short throughout the winter period. The efficiency of the workers and the standard of hygiene were satisfactory. Each worker was given a different disinfectant at each test and, in view of the number (376) of swabs involved, it is considered that the tests were made under comparable conditions.

During the summer there appeared to be little difference in the efficiency of the three disinfectant solutions and all three gave slightly better results than the water control. The efficiency of the individual worker appears to be a much more important factor in obtaining a clean udder prior to milking. Price (1938) found that scrubbing was the most important factor in removing the transient or contaminating bacterial flora from the skin; but removal of the normal or resident flora was somewhat more difficult.

In winter the advantage of the slightly better results obtained with hypochlorite at 600 p/m was probably offset by the irritant effect of the solution at this strength on the skin of the teats, udder and workers' hands. The QAC showed slightly better results in winter than the hypochlorite when both were used at 200 p/m, except in the control of sporeforming rods. However, over the whole period of the investigation there was little difference in the efficiency of the three disinfectants.

The results of the coli-aerogenes tests showed that hypochlorite at 600 p/m or even 200 p/m was more effective than the QAC at 200 p/m in eliminating these bacteria. The majority of those present were nonfaecal types as judged by the absence of growth at 44° in bile salt broth, less than one third of the isolates being 44° positive. The slightly higher incidence of 44° positives in summer is of interest, since various investigators have shown that *Klebsiella* spp. (44° negative) are the dominant coli-aerogenes bacteria in milk in summer. The dominance of these in milk in summer is presumably a temperature effect, as suggested by Malcolm (1933).

The proportions of the various types of organisms which survived differed only slightly between the four groups. In all cases, and particularly in the summer period, the surviving bacteria were mainly micrococci. A more serious question is raised by the fact that a high percentage of these surviving micrococci were coagulase positive staphylococci and hence possible causes of mastitis. The significance of these staphylococci in the latter connection is a matter for speculation. Innes (1956), Tee (1957) and Walters (1959) have drawn attention to the significant numbers of haemolytic staphylococci in bulk milk.

Few streptococci were found in any period. This, together with the small numbers of coli-aerogenes bacteria, indicates that either type of disinfectant is of value in reducing significantly the numbers of those organisms likely to affect the keeping quality of milk.

Examination of the Gram-negative rods showed that of the 116 cultures 80 caused no visible change in litmus milk and 17 gave an alkaline reaction. These 97 Gram-negative cultures were uniformly distributed through the four groups and there was no suggestion that a greater survival occurred with the QAC disinfectant. Of the 659 Gram-positive rods isolated only 5 were catalase negative and therefore possibly *Lactobacillus* spp. There was a much higher incidence of sporeforming Gram-positive rods in winter, particularly with the QAC group of cows. This was probably due to the increased amount of bedding and fodder present, but the results also suggest that the bacterial spore is more resistant to QACs. The results of the examination of the bacterial flora from this series of tests were comparable to those found by McKenzie & Rosborough (1958).

The results confirm the findings of the previous investigation, namely that there is little difference between the efficiency of the two types of disinfectant when used at a concentration of 200 p/m. The preference of the dairy workers, however, was for the hypochlorite solution at this strength. At 600 p/m, hypochlorite may have an irritant effect both on the skin of the teats and on workers' hands, especially if any cuts are present; but this will vary with the amount of free caustic alkalinity.

The main objection to the QAC disinfectant was that the teats felt slimy to the touch and the teat cups fell off more readily. Although the water supply on the farm was soft (17 p/m of permanent hardness) it should be noted that other investigators have found that QACs may not give satisfactory disinfection with hard water. Because the available chlorine in the first pail of hypochlorite wash water declined after washing six cows, this disinfectant must be renewed after every 6–8 cows. A second pail of water containing hypochlorite with a second cloth for drying the udder and teats is of considerable value.

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SOME PHENOL DECOMPOSING STRAINS OF *PSEUDOMONAS*

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SUMMARY: Three strains of *Pseudomonas* Migula, capable of utilizing phenol and other aromatic compounds as sole sources of carbon, are described. They resemble certain strains of *Ps. aeruginosa* and *P. fluorescens* except in this ability.

Many strains of *Pseudomonas* have been reported to have the ability to attack various aromatic compounds. These have sometimes been referred to *Ps. fluorescens* or, though less often, to *Ps. aeruginosa*. Sometimes they have been described simply as '*Pseudomonas* sp.' and occasionally they have been given the status of new species but seldom have they been fully characterized.

Two cultures of Gram-negative, nonsporing, polar flagellate rods were isolated from solutions of mineral salts to which 0·5% of phenol had been added as a preservative and from which it had completely disappeared. Both strains were able to grow for many successive subcultures in a mineral medium with 0·1% of phenol as the sole carbon source, and retained this ability even after prolonged culture in broth devoid of phenol. They produced pyocyanin as well as the fluorescent pigment common to many pseudomonads, and thus resembled *Ps. aeruginosa* (Schroëter Migula rather than any species particularly characterized by the ability to attack aromatic compounds, e.g. *Ps. phenolis* Hamdy *et al.* (1956). Further, one of 12 nonpyocyaninogenic cultures of *Pseudomonas* spp. from various sources, all of which had been maintained in broth for three or more years, was also found to be able to utilize phenol, and it seemed desirable to compare these cultures with strains of known species of *Pseudomonas* which did not possess that property.

Materials and Methods

Organisms. Three phenol decomposing strains were examined. Two of these were the new pyocyaninogenic isolates N 62 and N 63 from the phenolized mineral salts solution, mentioned above, and the third, G 489, was a nonpyocyaninogenic culture received from the Royal Technical College, Glasgow, as *Pseudomonas fluorescens*.

Three cultures which did not decompose phenol were also examined, namely G 497, a nonpyocyaninogenic strain received from the Royal Technical College Glasgow, as *Ps. fluorescens*; NCIB 5940, received as *Ps. fluorescens* from the Nationa

Collection of Industrial Bacteria and nonpyocyaninogenic; and N 5, originally isolated from a human lesion and referred to as *Ps. aeruginosa* because it was pyocyaninogenic, though it has since lost that property.

Identification procedures. The criteria suggested by Haynes (1951) for the identification of *Ps. aeruginosa* were first applied, and followed by a series of conventional tests. The Frazier (1926) plate method was used for detecting gelatin hydrolysis, 5% milk in tap water agar for casein hydrolysis, and tributyrin agar (Oxoid) and the olive oil plate method of Bulder (1955) for the detection of lipolytic activity.

Action on carbohydrates, organic acids, urea and other substances was examined in a mineral basal medium. Solutions in distilled water, sterilized separately by filtration, were added to give suitable concentrations (% w/v for solids and v/v for liquids) in a sterile mineral basal medium containing, all as % w/v in glass distilled water, $NH_4H_2PO_4$, 0·1; KCl, 0·02; $MgSO_4.7H_2O$, 0·02. Filter paper strips partly immersed in the basal medium and in peptone water were used to determine ability to decompose cellulose. The reaction of each medium was adjusted to pH 7·2–7·4 with sterile NaOH solution and the media, distributed into sterile tubes, were incubated for 7 days at 25° before use. This was the incubation temperature also, except where otherwise stated. Six replicate cultures of each organism in each medium were inoculated, each with 1 loopful from a suspension in sterile saline of cells from an 18 hr nutrient agar slope. These were examined at intervals up to 14 days, and tubes which showed definite turbidity as compared with control cultures in the basal medium without the carbon source were subcultured into further tubes of the same medium. Only if all replicates grew in three or more successive subcultures was a test recorded as positive, cultures which grew for only one or two generations being assumed to have utilized nutrients carried over with the inoculum. If the replicates varied, two or more continuing to grow in subcultures, the result was recorded as variable. With media containing sugars, alcohols or organic acids the final pH was determined before the cultures were discarded. Starch medium cultures (0·001% in the basal medium) were tested with iodine after 28 days and compared with a known starch positive control. Signs of cellulose decomposition were looked for after two months incubation.

RESULTS AND DISCUSSION

Cultures N 5, N 62, N 63 and NCIB 5940 all grew well in broth at 42° but failed to grow at 2°, and whereas NCIB 5940 grew very slowly at 5° the other three made no growth at all. In contrast, G 489 and G 497 grew well at the lower temperatures but failed to grow at 37°. All the cultures produced a reducing substance from potassium gluconate in shaken culture and formed copious slime on subsequent standing, but these two tests gave positive results with every *Pseudomonas* examined. All six cultures grew well in sea water broth and in MacConkey's broth, and all produced ammonia from urea (2% in the basal medium with KH_2PO_4 substituted for the ammonium salt). None produced indole or H_2S from peptone, and none attacked starch or cellulose.

Cultures N 5, N 62 and N 63 reduced nitrate to nitrite, liquefied coagulated serum, actively hydrolyzed gelatin, casein, tributyrin and olive oil and also haemolyzed human red blood corpuscles. Cultures N 62 and N 63 produced H_2S in Kligler's medium, but N 5 did not. Culture NCIB 5940 gave the same results as N 5, except that it failed to liquefy serum and was less active in hydrolyzing casein: but strains G 489 and G 497 gave negative results in all these tests.

All six cultures grew well and produced an acid reaction (pH 6·0 or less) in the basal medium plus 1% of glucose, fructose, mannose, xylose, glycerol and ethanol and failed to grow with 1% of sucrose, maltose or lactose; but whereas G 489 and G 497 grew and produced acid from cellobiose but failed to grow with mannitol (both 1%), N 5, N 62, N 63 and NCIB 5940 gave the reverse result. None of the cultures grew or produced any change of reaction with salicin (0·1%). With formic, acetic, citric, malic and lactic acids (0·1%), and with benzoic acid (0·05%), all six cultures grew well and produced a marked change of reaction to pH 8·0–8·5. But tartaric acid (0·1%) was utilized only by G 489 and G 497, and oxalic acid (0·1%) only by G 489, which grew poorly but produced a marked alkaline reaction.

These results suggest that the six cultures fall into two distinct groups, one comprising the two strains G 489 and G 497 and the other the four strains N 5, N 62, N 63 and NCIB 5940. Of the latter group, N 5, N 62 and N 63 can all be referred to *Ps. aeruginosa*, for N 5 was originally pyocyaninogenic though it has since lost that character. Strain NCIB 5940, which was presumably nonpyocyaninogenic even when first isolated, otherwise resembles N 5 except for its somewhat weaker proteolytic activity, and both these characters are notoriously unstable. The production of H_2S in Kligler's medium is the only other character in which the new isolates N 62 and N 63 differ from N 5 and NCIB 5940. Cultures G 489 and G 497, however, which are almost identical with each other, are distinct in several respects from the other four. They differ somewhat from *Ps. fluorescens* as described in the 7th edition of Bergey's *Manual* (Breed, Murray & Smith, 1957) but agree, in respect of the properties tested, with that species as defined by Rhodes (1959).

Despite this clear separation of the cultures into groups of two and four, the results of the tests of ability to grow in the basal medium with various aromatic compounds as sole carbon sources put them into a different arrangement. Cultures G 497, N 5 and NCIB 5940 form one group with little or no ability to attack these substances, and G 489 and the new isolates another, with considerable activity, as shown in Table 1.

These results emphasize the difficulty of defining species of *Pseudomonas* in terms of any arbitrary selection of biochemical characters. Presumably N 5, N 62 and N 63 must be referred to *Ps. aeruginosa* and G 489 and G 497 to *Ps. fluorescens*, but NCIB 5940 is more difficult to place. The difference between the two species is far from clear, as has been shown by Gaby (1946, 1955) and by Rhodes (1956, 1959). Both species can apparently produce strains able to attack aromatic compounds.

The ability to attack unusual substrates is commonly an adaptive character, but no clear evidence of adaptation was shown here. Although the cultures were maintained on meat extract media, each culture which could attack any given substrate did so on first inoculation into that substrate, producing distinct turbidity in 24 hr at 25°. Further subcultures in the same medium showed no apparent increase in rate or abundance of growth. For example, G 489 and G 497 attacked tartaric acid,

Table 1. *The abilities of six strains of* Pseudomonas *to grow in mineral basal medium with aromatic compounds as sole sources of carbon*

Aromatic carbon source†	Growth* of culture no.					
	N 5	N 62	N 63	NCIB 5940	G 489	G 497
Phenol	—	++	++	—	++	—
o-Cresol	—	++	++	—	±	—
m-Cresol	—	++	++	—	(+)	—
p-Cresol	—	+	+	—	(+)	—
2:3-Xylenol	—	±	±	—	—	—
2:5-Xylenol	—	—	—	—	—	—
2:6-Xylenol	—	—	—	—	—	—
3:4-Xylenol	—	—	—	—	—	—
3:5-Xylenol	—	(+)	(+)	—	—	—
Catechol	±	+	+	±	+	±
Resorcinol	—	(+)	(+)	—	±	—
Quinol	—	—	—	—	—	—
Pyrogallol	—	—	—	—	—	—
p-Hydroxybenzoic acid	±	+	+	±	(+)	—
Salicylic acid	—	—	—	—	(+)	—
Vanillin	±	+	+	—	+	—
Naphthalene	—	—	—	—	—	—

* Growth: ++, good; +, moderate; (+), slight growth in all replicates for three or more generations of subculture; ±, growth for three or more generations in some but not all replicates; —, no growth, or very slight growth in the first subculture only.

† Concentrations, 0·0025% (w/v), except for phenol, 0·1%, vanillin, 0·05% and naphthalene, *c.* 0·003%.

and the former produced its maximum (rather slight) turbidity in oxalic acid, in 24 hr. Later subcultures were no more turbid than the first, and attempts to induce the other cultures to attack these acids failed. Similarly, the phenol negative cultures could not be induced to attack most of the other aromatic compounds. They utilized benzoic acid readily, and could apparently produce variants able to attack catechol (all three cultures), *p*-hydroxybenzoic acid (N 5 and NCIB 5940) and vanillin (N 5 only). In these instances growth occurred in some of the replicate cultures of the first generation in these media (all replicates of the phenol positive cultures grew well) and positive cultures could be further subcultured, whereas attempts to subculture from the negative tubes failed.

The fact that all the phenol positive cultures, and even some variants of N 5 and NCIB 5940, attacked both catechol and *p*-hydroxybenzoic acid may be of interest, for two chief metabolic pathways, in which these two compounds are respectively the first steps, have been suggested for the microbial metabolism of phenol. Possibly some strains may be able to follow either pathway, the one actually chosen in any given instance depending upon other factors.

B. B. Davey wishes to thank the East Midlands Divisional Coal Board for permission to publish this note. His present address is: Products Research Department, Rowntree & Co. Ltd., York.

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A NOMOGRAPH FOR DETERMINING FIFTY PER CENT ENDPOINTS

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SUMMARY: A universal nomograph is presented for determining the 50% response dose (RD_{50}) from the cumulative percentage response. Instructions are given for the preparation of simpler nomographs for particular test conditions.

The method of Reed & Muench (1938) for estimating 50% endpoints has been widely applied in microbiological assay. To facilitate the use of this method in a continually enlarging assay programme, an abbreviated procedure for determining the 50% endpoint has been adopted in our laboratories. This report describes the construction and application of a nomograph for determining the 50% response dose (RD_{50}), based on the Reed & Muench method. It is presented in a form which makes it particularly applicable to infectivity and toxicity titrations, where percentage response decreases with increasing dilution: but it may be readily adapted for inhibition titrations.

Construction of the Nomograph

The nomograph is presented in Fig. 1. Cumulative percentage response is plotted on an arithmetic horizontal scale, with the level dropped at the 50% point (AB, CD). At the 50% point, vertical arithmetic and logarithmic scales are inserted (CB). For the arithmetic scale, the distance CB is set as one unit and appropriate subdivisions are made. This scale represents proportional distance. The logarithmic scale, from 1 to 10, gives the antilogarithms of the corresponding values on the arithmetic scale.

Use of the Nomograph

The applications of the nomograph are presented in a natural sequence, to provide a clear understanding of its functions, and for the sake of clarity the data from three sample titrations, given in Table 1, are used.

Determination of proportional distance. The cumulative percentage responses for a titration having been determined (Table 1), a straight edge is used to join the cumulative percentage mortality next below 50 (scale AB) with the value next above 50 (scale CD) and the proportional distance is read on the arithmetic scale at the point of intersection with BC. For example, in titration 1, 13·3% on scale AB is joined to 61·5% on scale CD. The point of intersection on scale BC, 0·24, is the proportional distance from 10^{-3}. Similarly, in titration 2 the proportional distance from $10^{-4\cdot5}$ is 0·16, and in titration 3 the proportional distance from 1:4 is 0·6.

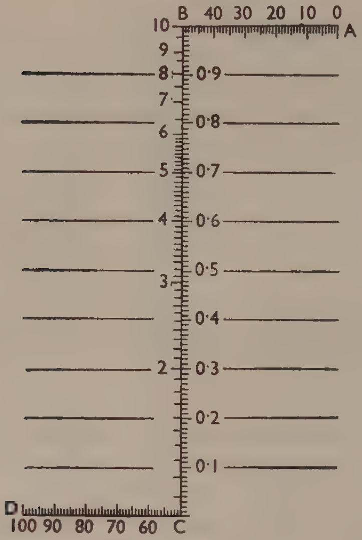

Fig. 1. Nomograph for determining RD_{50} from cumulative percentage response.

Determination of proportional distance times log dilution factor. For this step, AB should be visualized as a sliding scale which may be moved to any level, but remains perpendicular to CB. The operation is performed by moving AB to the position representing the logarithm of the dilution factor, and repeating the process described above for determining proportional distance.

Table 1. *Data obtained in sample titrations for determining* RD_{50}

Titration no.	Dilution	Response		Cumulative response			RD_{50}	
		+ve	−ve	+ve	−ve	%	Nomograph	Calculated
1	10^{-1}	10	0	27	0	100·0		
	10^{-2}	9	1	17	1	94·4		
	10^{-3}	6	4	8	5	61·5	$10^{-3·24}$	$10^{-3·24}$
	10^{-4}	2	8	2	13	13·3		
	10^{-5}	0	10	0	23	0·0		
2	$10^{-3·5}$	5	0	13	0	100·0		
	$10^{-4·0}$	4	1	8	1	88·9		
	$10^{-4·5}$	3	2	4	3	57·1	$10^{-4·58}$	$10^{-4·58}$
	$10^{-5·0}$	1	4	1	7	12·5		
	$10^{-5·5}$	0	5	0	12	0·0		
3	1:1	10	0	31	0	100·0		
	1:2	9	1	21	1	95·5		
	1:4	7	3	12	4	75·0		
	1:8	4	6	5	10	33·3	1:6	1:6
	1:16	1	9	1	19	5·0		
	1:32	0	10	0	29	0·0		

In titration 1, the logarithm of the dilution factor is 1, and the procedure is identical with that used for determining proportional distance. In titration 2, the logarithm of the dilution factor is 0·5. When B is located at 0·5, the line joining 57·1% and 12·5% intersects BC at 0·08, and this is the proportional distance times the logarithm of the dilution factor. Serial twofold dilutions were used for titration 3, and when B is located at 0·3, the logarithm of the dilution factor, the line joining 75·0% and 33·3% intersects BC at 0·18.

Where dilutions are expressed as powers of 10, the RD_{50} may be determined by subtracting the value obtained for proportional distance times log dilution factor from the exponential of the lower dilution. Thus, in titration 1, the RD_{50} is $10^{-3-0\cdot24}$, or $10^{-3\cdot24}$. Similarly, the RD_{50} for titration 2 is $10^{-4\cdot5-0\cdot08}$, or $10^{-4\cdot58}$.

Determination of RD_{50} by direct reading. This application is particularly useful when serial dilutions are expressed as fractions. In it one must visualize both AB and CD as sliding scales and locate C and B at the positions representing the dilutions between which the 50% point lies, using the logarithmic scale. In titration 3, the RD_{50} lies between 1:4 and 1:8, so scale CD is raised to 4 and scale AB lowered to 8. With these positions, the line joining 75·0% and 33·3% intersects BC at 6 and the RD_{50} is 1:6.

Extension of the logarithmic scale for one additional cycle beyond B would facilitate direct reading. For example, with an RD_{50} between 1:8 and 1:16, the AB scale could be raised to 16. Without this extension, the RD_{50} is determined by working in fractions of the real values. Thus, if the RD_{50} lies between 1:8 and 1:16 the determination is made for an RD_{50} between 1:4 and 1:8 and the result is multiplied by 2. Similarly, if fivefold dilutions are used and the RD_{50} falls between 5 and 25, the determination is made between 1 and 5 and the value obtained multiplied by 5.

Reliability of the nomograph for determining proportional distance. As the precision of the nomograph depends on the number of subdivisions available in the portion of BC used, it varies with the application. The reliability of the reading is a reflection of the accuracy with which proportional distance may be determined, and this can be illustrated by the results of a set of 20 nomographic determinations of the proportional distance, in which agreement with the calculated value was exact in 13 cases, and in the other 7 the difference was only ±0·01. The standard error of the estimate was 0·0084.

DISCUSSION

The nomograph has been described in a manner which indicates its applicability for any dilution series. This involves the visualization of sliding scales, but for each specific set of test conditions a nomograph with stationary scales may be rapidly constructed on regular graph paper. The nomograph presented in Fig. 1, without the logarithmic scale, is adequate for all titrations involving serial tenfold dilutions, but for other dilutions expressed in powers of ten the distance CB is set equal to the logarithm of the dilution factor. Nomographs of this type may be constructed on arithmetic graph paper, but when dilutions are expressed as fractions the nomograph is constructed on 2 cycle semilogarithmic paper, one cycle of the logarithmic scale being used for CB and the other extending beyond B. For convenience, cumulative percentage response scales may be marked at each dilution level used in the test.

The nomograph is in the form applicable to titrations where response decreases with increasing dilution: but when inhibitors are titrated, response increases with increasing dilution and the nomograph may be adapted for use in these circumstances by reversing the positions of AB and CD.

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THE PRESERVATION OF MARINE BACTERIA

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SUMMARY: Forty-five marine bacteria and five nonmarine strains, representing 10 genera, were preserved by freeze drying, by storage in glycerol-nutrient broth at −29° and under paraffin oil at 1°. Checks on viability and viable counts were made over a two year storage period. At the end of this time 100% of the strains had been preserved by freeze drying, 86% by storage in glycerol-nutrient broth at low temperature and 72% under paraffin oil. The greatest loss of viability occurred among the vibrios and spirilla whichever of the three methods was used, but coryneforms, achromobacters and micrococci were successfully preserved by all these methods.

Since most of the work on the preservation of bacteria has been concerned with nonmarine organisms it was necessary, when setting up the National Collection of Marine Bacteria (NCMB), to find out if bacteria from the sea can be preserved by the same techniques. It was decided therefore to study the suitability for this purpose of freeze drying, of freezing in glycerol-nutrient broth and of storage under paraffin oil, and to follow, as far as was practical, the fate of the organisms during a two year storage period. Moreover, such a study would make it possible to compare the effectiveness of the methods which would be valuable in itself, because although freeze drying and other means of preserving bacteria have been in general use for some time there have been few contributions to the literature in which comparisons have been made between the various preservation techniques. It was also decided to enhance the usefulness of the study by making it as quantitative as possible, for in spite of some valuable papers, such as those of Proom & Hemmons (1949), Fry & Greaves (1951) and Hörter (1958, 1960), much is still unknown of the changes in bacterial count which occur during the preservation process or during subsequent storage.

Methods and Materials

Cultures. Forty-five marine bacteria representing 10 genera were used in the investigation. These organisms had been originally isolated by several different workers from marine fish, sea water, sea mud and other marine environments in the Arctic Ocean, the North Sea, the eastern Pacific Ocean and the Gulf of Mexico. Five nonmarine organisms, *Pseudomonas aeruginosa* (NCIB 6570 and 950), *Ps. oleovorans* (NCIB 6576), *Ps. formicans* (Crawford, 1954) and *Aeromonas hydrophila* (NCTC 7810) were included, to enable comparisons to be made between marine and nonmarine strains and as a control on the techniques. Most of the marine isolates were characterized only as far as the genus. The achromobacters, aeromonads, pseudomonads and vibrios were identified using the techniques of Shewan, Hobbs &

Hodgkiss (1960). Five organisms, NCMB 1, 2, 7, 24 and 25, were luminous and were originally placed in the genus *Photobacterium*, but have been reclassified as *Aeromonas* spp. or *Vibrio* spp. by Spencer (1955). Although all the marine organisms were isolated on sea water media, it was found that after a few transfers most of them grew very well on tap water nutrient agar. This medium was used for growing all such adapted strains. However, 17 cultures (see Table 1) could not be so adapted and were grown on sea water nutrient agar.

Preservation methods

Freeze drying. The cells were usually harvested after incubation for 3 or 4 days at their optimum temperature, that is towards the end of their logarithmic growth phase, as there is some evidence that older cultures survive freeze drying better than young ones (Fry & Greaves, 1951; Fry, 1954; Toyokawa & Hollander, 1956). A thick suspension of the cells was then made in 'Mist desiccans' (nutrient broth 1 part and horse serum 3 parts, containing 7·5% (w/v) of glucose), and 0·1 ml amounts pipetted into ampoules which had been previously prepared by being soaked overnight in 5% HCl, rinsed thoroughly, dried and sterilized. Each ampoule also contained a piece of filter paper on which the identification of the organism and the date had been stamped, using 'ENM' ink (Gutteridge Sampson Ltd., 155, Farringdon Road, London, E.C.1) which is known to be harmless to bacteria. Freeze drying was carried out in an LC5 centrifugal freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex). Primary drying was carried on for 1–2 hr, and secondary drying for 16–20 hr. The ampoules were sealed off at a pressure of 0·012–0·028 mm Hg, as measured by a Pirani gauge, and stored at room temperature.

Storage in glycerol-nutrient broth. Several workers (Hollander & Nell, 1954; Howard, 1956; Fox & Hotchkiss, 1957; Quadling, 1960) have shown that the presence of glycerol in a medium reduces the number of bacteria which are killed when the suspension is frozen. In this study cultures which had been growing for 3 or 4 days on tap water nutrient agar or sea water nutrient agar were preserved, using the glycerol-nutrient broth method of Howard (1956). The cells were harvested, washed with normal saline or sea water, suspended in nutrient broth to which 15% (v/v) of sterile glycerol had been added, and stored at –29°.

Storage under mineral paraffin oil. Mineral paraffin oil was first employed for preserving cultures by Lumière & Chevrotier (1914) and has been successfully used by many workers since then. Recently this method has been re-examined by Hartsell (1953, 1956) and Rhodes (1956, 1957). For the present investigation the organisms were grown on short, deep slopes for 2 or 3 days until there was abundant growth. This was then covered with a layer of sterile paraffin oil (B.P. quality), care being taken to cover the slope completely. The cultures were stored at 1°.

Viability checks

Freeze drying method. Using the Miles & Misra (1938) technique, the numbers of bacteria were determined in the 'Mist desiccans' suspension shortly before drying and in the dried material immediately after sealing. Further counts were made after storage for 1, 3, 6, 12 and 24 months.

It is known that the method and speed of resuscitation may have a profound effect on the recovery of freeze dried cultures (Wasserman & Hopkins, 1957; Leach & Scott, 1959). The method of resuscitation used here was to add 0·5 ml of tap water nutrient broth or sea water nutrient broth to the contents of an ampoule and to resuspend the freeze dried material as rapidly as possible.

Glycerol-nutrient broth and paraffin methods. The viability of the organisms preserved by the glycerol-nutrient broth and paraffin methods was checked by streaking one loopful on a tap water nutrient agar or sea water nutrient agar plate after 1, 3, 6, 12 and 24 months. In the case of the glycerol-nutrient broth method a fresh tube, i.e. one that had not previously been thawed, was taken for each check; in the case of the paraffin method, all the samples were taken from the same slope.

Results and Discussion

Freeze dried cultures

The bacterial counts on the freeze dried material are shown in Table 1: they are expressed as the logarithm to base 10 of the number of organisms/ml of 'Mist desiccans'. There was a much greater variation in the bacterial counts than could be accounted for by the inherent statistical error of the Miles & Misra method, and this was believed to be due to the excessive clumping that occurred when the organisms, particularly the *Achromobacter* spp., were mixed with the 'Mist desiccans'. This effect was so marked in the case of NCMB 138 that the organism formed a precipitate and settled at the bottom of the ampoule. In other cases the effect was less marked, but could be observed by mixing the organism with 'Mist desiccans' on a microscope slide. These aggregations were not easily dispersed; even continuous flushing into and out of a Pasteur pipette for 20 min failed to break up all the clumps in resuscitated cultures. The cause of this excessive clumping is not known, but it has been observed occasionally particularly among certain slime producing genera such as *Azotobacter* (MacKenzie, pers. comm.). These aggregations were also reminiscent of those formed by rough strains of coli-aerogenes bacteria in low salt concentrations (Mitchell, 1951), although the colonies appeared to be smooth. The effect would be explained if it could be shown that marine bacteria have a strongly hydrophobic surface, or that other cohesive forces which exist between the cells came into play when the bacteria were mixed with 'Mist desiccans'. Further work on the action of electrolytes or colloids in various concentrations on these cohesive forces would be valuable. Undoubtedly the clumping accounted for some unexpectedly low counts, particularly in the case of NCMB 131 and 138. Moreover, the degree of clumping made a statistical evaluation of the results impracticable. In spite of these difficulties, however, certain conclusions may legitimately be drawn from the results. First, although in some cases there was a considerable reduction in count, freeze drying successfully preserved marine as well as nonmarine organisms. That the requirement of some of the organisms for salt for growth is not an important factor in their susceptibility to freeze drying is shown by the fact that several of the organisms requiring sea water showed no marked loss of viability when freeze dried whereas others were killed off to a considerable extent. Within the limits imposed by the

Table 1. *The influence of freeze drying in 'Mist desiccans' and storage at room temperature on the survival of various bacteria*

Identification	Culture collection* and number	Log$_{10}$ (colony count/ml of 'Mist desiccans')						
		Before drying	After drying and storage for (months)					
			0	1	3	6	12	24
Achromobacter spp.	NCMB 9†	8·3	8·0	7·9	7·5	7·3	7·6	7·0
	NCMB 20†	8·3	6·3	5·6	5·7	6·8	5·5	6·9
	NCMB 26	9·5	9·4	8·5	8·0	9·4	8·3	8·5
	NCMB 27	7·8	6·4	6·1	8·3	8·0	7·8	8·0
	NCMB 28	8·8	8·0	8·7	8·0	8·9	8·0	8·8
	NCMB 29	8·9	8·8	9·0	9·0	9·4	8·7	7·4
	NCMB 131	9·7	6·7	6·7	8·0	6·7	9·2	9·0
	NCMB 132	10·0	8·5	8·5	6·2	9·0	9·4	8·0
	NCMB 135	9·6	10·0	9·8	10·0	9·7	9·7	9·0
	NCMB 138	10·5	9·2	7·9	9·7	9·9	7·0	8·8
Aeromonas harveyi	NCMB 2†	9·1	7·0	6·5	6·2	5·9	6·5	6·4
Aer. sepiae	NCMB 24†	10·3	7·8	7·1	7·6	7·8	6·0	5·3
Aer. hydrophila	NCTC 7810	10·8	9·9	8·5	7·0	7·8	7·3	7·0
Corynebacterium erythrogenes	NCMB 5	9·8	9·7	9·7	9·8	9·8	9·7	9·5
Corynebacterium sp.	NCMB 8	10·1	9·6	9·8	10·1	9·8	10·2	10·3
	NCMB 12	9·8	9·8	9·6	9·7	9·5	9·7	9·4
	NCMB 16†	9·3	9·3	9·3	9·3	9·8	9·0	9·0
	NCMB 31	10·4	10·7	9·8	9·7	10·0	9·9	9·9
	NCMB 32	10·5	9·6	10·0	9·7	9·4	9·3	9·3
	NCMB 33	10·7	10·2	10·0	9·8	9·9	10·5	10·6
	NCMB 34	10·2	9·5	9·5	9·7	9·2	9·8	9·3
	NCMB 35	10·6	9·5	9·5	9·6	9·3	9·8	10·0
	NCMB 39	11·4	11·3	10·5	10·6	10·4	10·4	10·2
Flavobacterium spp.	NCMB 21	8·9	8·6	8·5	8·7	9·3	9·2	9·2
	NCMB 22†	6·8	6·4	5·8	6·7	6·8	6·0	6·3
Micrococcus spp.	NCMB 13	10·0	9·9	9·6	9·7	9·8	9·8	10·0
	NCMB 14	10·6	10·1	10·5	11·0	10·0	11·0	10·0
	NCMB 15	9·7	9·7	9·6	9·7	9·8	9·3	9·3
Myxobacterium sp.	NCMB 11†	9·5	9·3	9·5	8·2	7·7	8·0	7·4
Pseudomonas aeruginosa	NCIB 6750	9·3	8·0	8·5	7·8	7·7	8·2	8·3
	NCIB 950	10·3	10·0	10·0	9·4	9·3	9·5	9·3
Ps. oleovorans	NCIB 6576	9·3	8·3	8·0	7·7	7·8	8·0	7·2
Pseudomonas spp.	NCMB 17†	7·3	5·5	5·0	5·0	5·6	5·3	5·4
	NCMB 18†	9·4	9·3	9·0	8·8	9·1	8·7	8·8
	NCMB 19†	9·4	8·3	8·3	7·0	8·6	6·3	8·0
Ps. formicans	NCMB 23	9·5	9·0	8·6	7·9	7·3	7·3	6·0
Pseudomonas spp.	NCMB 125	9·3	8·6	8·5	8·7	8·0	8·8	8·4
	NCMB 126	5·7	5·4	5·7	5·7	6·7	6·4	6·3
	NCMB 127	9·8	8·7	9·0	9·7	9·0	9·6	8·2
	NCMB 128	10·4	9·9	9·5	9·6	9·3	9·9	8·8
	NCMB 133	10·3	9·4	9·7	9·5	9·7	10·0	9·3
	NCMB 136	9·7	9·3	9·2	9·2	9·8	9·7	8·6
Serratia marinorubra	NCMB 4	10·1	9·3	8·9	9·4	9·2	9·5	9·3
Spirillum spp.	NCMB 38†	10·3	8·2	8·2	8·1	8·7	8·7	8·4
	NCMB 40†	10·4	6·7	7·6	6·3	7·6	6·3	3·5
Vibrio splendidum	NCMB 1†	7·0	4·5	4·4	4·3	3·8	4·3	4·7
V. anguillarum	NCMB 6†	9·2	6·3	5·0	5·9	6·0	6·0	5·4
V. phosphoreum	NCMB 7†	8·3	4·9	4·0	5·3	5·3	5·2	4·5
V. pierantonii	NCMB 25†	9·8	4·0	4·2	5·3	5·5	4·8	4·3
Vibrio sp.	NCMB 36†	10·2	7·5	7·7	7·5	7·6	7·3	5·5

* NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria; NCMB, National Collection of Marine Bacteria. † Require sea water for growth.

number of strains used, it is clear that the corynebacteria, flavobacteria and micrococci were resistant to freeze drying, the count dropping on an average by less than one third. The pseudomonads and achromobacters showed a greater strain to strain variation and in general the decrease was by a factor approaching one tenth. The greatest losses during freeze drying occurred among the spirilla and vibrios and, to a lesser degree, among the aeromonads. The high susceptibility of *Vibrio* spp. to freeze drying has been reported several times (e.g. Fry, 1954) and it may well be a feature of this genus.

Generally speaking the strains lost little or none of their viability during storage, although there were clear exceptions such as NCMB 23, 36, and 40. Cultures which markedly decreased in numbers during freeze drying did not necessarily die off to any extent during storage, whereas some organisms, which withstood freeze drying

Table 2. *The survival of bacteria after storage in glycerol-nutrient broth at −29° (a) and under mineral paraffin oil at 1° (b)*

Identification	Culture collection† and number	Viability* after storage for (months)									
		1		3		6		12		24	
		(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Achromobacter spp.	NCMB 9	+	++	+	++	+	++	+	++	+	++
	NCMB 20	++	+	++	+	++	+	++	+	++	+
	NCMB 26	++	++	++	++	++	++	++	+	+	+
	NCMB 27	++	++	++	++	++	++	+	+	+	+
	NCMB 28 NCMB 29 NCMB 131	++	++	++	++	++	++	++	++	++	++
	NCMB 132 NCMB 135 NCMB 138	++	++	++	++	++	++	++	++	++	+
Aeromonas harveyi	NCMB 2	+	++	+	+	+	−	−	−	−	−
Aer. sepiae	NCMB 24	++	++	++	+	+	−	+	−	+	−
Aer. hydrophila	NCTC 7810	++	++	++	++	++	++	+	+	+	−
Corynebacterium erythrogenes *Corynebacterium* spp.	NCMB 5 NCMB 8 NCMB 12 NCMB 16 NCMB 31 NCMB 32 NCMB 33 NCMB 34 NCMB 35 NCMB 39	++	++	++	++	++	++	++	++	++	++
Flavobacterium spp.	NCMB 21	++	++	++	++	++	++	++	++	++	++
	NCMB 22	++	++	++	++	++	++	+	+	+	−
Micrococcus spp.	NCMB 13 NCMB 14 NCMB 15	++	++	++	++	++	++	++	++	++	++
Myxobacterium sp.	NCMB 11	++	++	++	+	++	+	++	−	++	−
Pseudomonas aeruginosa	NCIB 6750	++	++	+	+	+	−	+	−	+	−
	NCIB 950	++	++	++	++	++	++	++	+	++	−
Ps. oleovorans	NCIB 6576	++	++	++	++	++	++	++	+	+	+
Pseudomonas spp.	NCMB 17	+	++	+	++	+	++	−	++	−	++
	NCMB 18 NCMB 19	++	++	++	++	++	++	++	++	+	−
Ps. formicans	NCMB 23	++	++	++	++	++	++	+	−	+	−
Pseudomonas spp.	NCMB 125 NCMB 126 NCMB 127	++	++	++	++	++	++	++	++	++	++
	NCMB 128 NCMB 133 NCMB 136	++	++	++	++	++	++	++	++	++	+
Serratia marinorubra	NCMB 4	++	++	++	++	++	++	++	++	++	++
Spirillum spp.	NCMB 38	++	++	++	++	++	++	++	++	−	+
	NCMB 40	++	++	+	+	+	−	+	−	−	−
Vibrio splendidum	NCMB 1	+	++	+	+	−	−	−	−	−	−
V. anguillarum	NCMB 6	++	++	++	++	++	++	++	++	++	++
V. phosphorerum	NCMB 7	++	++	+	++	+	++		+	−	+
V. pierantonii	NCMB 25	++	++	++		++	−	+	−	−	−
Vibrio sp.	NCMB 36	++	−	++	−	++	−	+	−	+	−

* Growth after subculture: ++, abundant to good; +, moderate to slight; −, none.

† NCTC, National Collection of Type Cultures; NCIB, National Collection of Industrial Bacteria; NCMB, National Collection of Marine Bacteria.

well, tended to die off during the 2 year storage period. Occasionally an organism appeared to increase in numbers during storage, as for example NCMB 27 and 126 and, to a less marked extent, NCMB 33 and 35. This rise may have been partly due to the experimental error within the counting method, but it is interesting to notice that Maister, Pfeifer, Bogart & Heger (1958) found similar increases in viable count of *Serratia marcescens* after storage of freeze dried pellets. They suggested that the increase may be due to the recovery of enzymic systems during storage or to the breaking up of clumps. Such a possibility should not be ruled out here.

Stored cultures

The viability of cultures preserved by the glycerol-nutrient broth method and under paraffin oil is shown in Table 2; 86% of the cultures preserved by the former method and 72% of those preserved by the latter method were viable after 2 years. It is interesting to note that in both cases, as in the freeze drying experiment, the vibrios, aeromonads and spirilla were the most easily killed of all the groups examined. In fact, both the most difficult and the easiest organisms to preserve were generally the same whichever process of preservation was used. Again there appeared to be no relationship between the requirement of sea water for growth and the effectiveness of preservation by either the glycerol-nutrient broth method or the paraffin method; marine organisms behaved in the same way as nonmarine ones. One unexpected result was the loss of the *Ps. aeruginosa* strains under paraffin oil. Hartsell (1953) was able to maintain this species for more than 9 years under paraffin; he was also able to preserve *V. comma* for more than 6 years. Gordon & Smith (1947), on the contrary, found the mineral oil method unsuitable for some strains of pseudomonads and vibrios. This variation in results may be due to storage methods; Hartsell (1953) stored his cultures at 25°, whereas in this investigation the storage temperature was 1°.

Conclusion

It may be concluded from these results that marine bacteria can be preserved by well established techniques. From the point of view of Culture Collections each method has advantages and disadvantages. The advantages of freeze drying are that the cells remain viable for a long time and that ampoules of freeze dried bacteria are light and easy to despatch; the disadvantage is that the process is slow and tedious. Both the mineral oil and glycerol-nutrient broth methods are rapid and cheap, but on the data presented here not as successful as freeze drying for preserving marine bacteria for a long time.

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THE EFFECT OF LOW TEMPERATURES ON THE INACTIVATION BY IONIZING RADIATIONS OF *CLOSTRIDIUM BOTULINUM* SPORES IN MEAT

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SUMMARY: The effect of temperature on the inactivation of *Clostridium botulinum* spores has been studied in minced pork meat irradiated with 2 MeV electrons and gamma rays. When electrons were used, differences in survival after 1 Mrad at 18°, 0° and −75° were small and were significant only when temperatures of 0° and 18° were compared, a slightly higher survival being obtained at 0°. The effects at temperatures of 0° and −75° were further compared, using a range of gamma ray doses, and regression lines were calculated for the inactivations. From these, 'sterilizing doses', i.e. those which would give a survivor level of 10^{-12}, were calculated, together with their 95% confidence limits. The errors involved in this type of estimate are discussed: it is concluded that there is no evidence of a significant difference in the computed sterilizing doses at 0° and at −75°.

The freezing of meat before irradiation greatly diminishes the off flavours and odours otherwise produced (Coleby, Ingram, Shepherd, Thornley & Wilson, 1961). However, as the dose required for a commercially acceptable degree of sterility in meat is based on that necessary to reduce the number of *Clostridium botulinum* spores by a factor of 10^{12} (Schmidt & Nank, 1960) it is necessary to determine whether irradiation at a low temperature has any marked influence on this dose requirement.

Denny, Bohrer, Perkins & Townsend (1959) irradiated *Cl. botulinum* spores in phosphate buffer at room temperature and in the frozen state, and obtained significantly greater survival at the lower temperature. Kempe, Bonventre, Graikoski & Williams (1956) also irradiated in phosphate buffer but at temperatures between −80° and +80°, and found the inactivation to be similar at all temperatures except +25°, at which there was considerably greater survival.

The principal conclusion of our experiments, that the temperature of irradiation had little influence on the calculated sterilizing dose, has already been stated briefly (Ingram, Coleby, Thornley & Wilson, 1959), but in the present paper the data are considered more fully, with experimental details.

Methods and Materials

Because the work of others (Denny *et al.* 1959) had shown that there is much greater inactivation when spores are irradiated in an inorganic buffer solution than in an organic material, it was necessary to make the experiments with meat itself, which was minced to facilitate the distribution of the spores.

Since the spores were to be distributed in meat, it was foreseen that there would be a difficulty in using normal temperatures for irradiation, because unless the exposure times were brief, germination, which could lead to greatly lowered resistance, might occur. This difficulty was avoided by using temperatures of 0° and −75° for the main part of the work, which was done with gamma rays and involved long exposures, and by doing a comparison of irradiations at 18°, 0° and −75° only at the largest irradiation dose which, with the facilities available, could be delivered by electrons within a time sufficiently short to avoid germination.

The general plan of the investigation was to add a measured amount of spore suspension to a small container of meat (chosen for its suitability for the irradiation), and to examine the whole contents, or an appropriate dilution thereof, to estimate the survivors. This procedure avoids the sampling errors likely to occur in selecting aliquots of infected mince, but necessitates the substantial replication of containers because of the variation which occurs, especially between irradiated samples. In the electron irradiation experiments 5 replicate cans were used for each treatment, but only 3 were used in the later gamma ray experiments.

Preparation of spore suspensions. Clostridium botulinum (Type A, NCTC 7272) was incubated for 3 weeks at 37° in Hartley's tryptic digest broth containing 0·3% of starch. After centrifuging, the organisms were washed twice with McIlvaine's citrate-phosphate buffer of pH 5·5, near that of the meat, and resuspended in that buffer. The first suspension prepared (A) was then heated at 75° for 30 min to kill vegetative cells and to provide the heat shock thought necessary for germination (Evans & Curran, 1943). The number of viable spores was then $1{\cdot}7 \times 10^7$/ml, and this suspension was stored at 1° and used for electron irradiations in experiments 1 and 2. Suspension B, which was used for experiments 3–5, was prepared in the same way, except that it was heated only to 65° for 15 min. It initially contained the same number of spores as suspension A, but the viable count decreased gradually during the period of 2 months in which these experiments were done and, for experiment 5, only $4{\cdot}8 \times 10^6$ spores/ml were viable. For experiment 6, therefore, the concentration was increased fourfold by centrifuging and resuspending.

Preparation of meat samples. To avoid the difficulty of distinguishing *Cl. botulinum* among casual contaminants surviving irradiation, it was advantageous to make the experiments in meat with an initially low bacterial count.

The meat was taken from the *longissimus dorsi* muscle of a pig killed the previous day, and it was prepared freshly for each experiment. A piece of loin weighing about 3 Kg was obtained from the slaughterhouse, the outer surfaces were thoroughly flamed with 75% alcohol and removed with sterile instruments. Then, using aseptic precautions, pieces of muscle were cut out, minced and packed into small aluminium cans. With this procedure, contamination was so infrequent that the experiments proceeded as though the *Cl. botulinum* spores were virtually in pure culture.

Containers. The cans, which were of lacquered aluminium 0·25 mm thick, were 3·1 cm in diameter. The base was 1 cm deep and, when the domed slip-on lid was in position, the total depth was 1·4 cm. For irradiation with 2 MeV electrons, from one side only, it was necessary to limit the thickness of the meat samples to 5 mm. This was done by pipetting molten paraffin wax containing 10% polythene into the base, so that when the wax had solidified the depth was nowhere more than 5 mm. During subsequent filling, the meat was levelled to ensure that no part was thicker than this. A head space of about 4 mm remained below the domed lid. This standard packing was used in all experiments, even with gamma rays.

For gas packing, a brogue hole was made in the lid and sealed with adhesive tape until required.

Inoculation. After the cans had been filled with meat they were chilled to 5° and then kept on ice, except for periods of less than 15 min during inoculation and gas-packing.

Inoculation was done with an 'Agla' micrometer syringe (Burroughs Wellcome Ltd.) which delivered 0·05 ml of spore suspension with a nominal error of ±0·00005 ml. The inoculum was introduced centrally near the bottom of the meat sample, but was seen to spread through it. The sides of the can were coated with vacuum wax (Everett's no. 2, soft) to seal the joint between lid and base. The can was gas-packed by evacuating to 5 cm of mercury and filling with oxygen-free nitrogen: this was done three times and the brogue hole then sealed with vacuum wax. After this the samples were left overnight at 0° so that any trace of residual oxygen might be consumed.

Irradiations. At least 2 hr before irradiation the sealed cans to be treated at −75° were transferred to solid carbon dioxide, the others being kept at 0°. During electron treatments, temperatures were controlled by placing the cans, in trays, on solid CO_2 for irradiation at −75° or on ice for irradiation at 0°, the trays being covered with polythene film. For irradiation at *c.* 18°, cans were transferred from 0° to a water bath at 18° for 10 min (a procedure which had been found to raise the internal temperature of the meat to 17·5°), irradiated, and immediately replaced in ice. As controls, a set of cans was taken through the same procedure at each temperature but without irradiation. In addition, a sample warmed to *c.* 18° was checked for germination of the spores by making counts on a suspension of its contents before and after heating to 75° for 15 min. Comparison of these with similar counts on a sample previously held at 0° showed that the proportion of cells surviving the heat treatment was the same in both cases.

For the electron irradiations a 2 MeV Van de Graaff generator was used, the cans being placed on a conveyor belt which carried them through the downward directed beam of electrons. It was calculated, from measurements of beam current and its geometrical distribution, that one passage under the beam gave a dose of 1 Mrad.

Gamma rays were provided by a spent fuel rod assembly. Cans were arranged down the centre of Dewar flasks and the free space packed with solid CO_2 or ice according to the temperature required. The flasks were enclosed in containers for immersion in the irradiation pool, and adequate amounts of solid CO_2 or ice remained in the flasks throughout irradiation. The dose rates varied from 0·93 to 1·86 Mrad/hr.

The calculated dosages, for either radiation source, were checked by ceric sulphate dosimetry (Taimuty, Fowle & Peterson, 1959), which indicated that the nominal doses needed correction only by a factor of 0·95±0·03, and the differences have been neglected in our calculations.

Immediately after irradiation the cans in solid CO_2 were put back in ice, and all cans were kept thus at 0° until sampled for bacterial counts. Where electrons were used sampling was done on the day of irradiation and the following day, but with gamma rays it was done on the two days following irradiation.

Viable counts. Each can contained approximately 3 g of meat. The whole was transferred to a wide mouthed 1 oz bottle containing 20 ml of diluent and glass beads, and shaken for 1 min. The diluent was quarter-strength Ringer's solution containing 0·1% of sodium thioglycollate. Serial tenfold dilutions were made, and 1 ml amounts were plated, in duplicate, in pork liver infusion agar containing starch (Olsen & Scott, 1946) and thioglycollate (Hirsch & Grinsted, 1954). The preparation of this medium was described by Ingram & Handford (1957).

Plates from all inoculated samples were incubated anaerobically at 37° for 48 hr. In each experiment counts were also made on the uninoculated mince, the plates being incubated anaerobically for 48 hr and then aerobically for the same period. The results were calculated as viable bacteria/g of mince, using counts at two different dilutions with weighting for dilution, as described by Farmiloe *et al.* (1954).

RESULTS

No colonies developed on the plates from the uninoculated mince, after the full period of anaerobic and aerobic incubation, and this indicated that it contained fewer than 10 viable bacteria/g. Also, since no alien colonies developed on plates from the inoculated mince after anaerobic incubation, the data are believed to refer only to the added *Cl. botulinum* spores.

Method of counting spores

The viable counts for experiment 1 with electrons are given in full in Table 1. This shows satisfactory agreement between duplicate plates of the same dilution, and

Table 1. *The effect of temperature on the survival of* Cl. botulinum *spores in minced pork irradiated with* 1 *Mrad of* 2 *MeV electrons*

(Data from expt 1, spore suspension A)

Irradiation temperature (°C)	Control series						Irradiated series						
	Trial no.	Colony count at a dilution of				Viable count of pork (10^5/g)	Trial no.	Colony count at a dilution of				Viable count of pork (10^4/g)	Survivors (% of control)
		10^{-2}		10^{-3}				10^{-2}		10^{-3}			
18	26	312	348	31	34	2·53	21	56	52	3	3	3·97	
	27	387	377	34	48	2·96	22	15	13	1	0	1·01	—
	28	333	337	47	31	2·61	23	28	32	2	1	2·20	—
	29	297	271	28	34	2·20	24	36	58	7	5	3·69	—
	30	301	283	24	14	2·17	25	42	52	5	7	3·58	—
		Geometric mean:				2·48		Geometric mean:				2·59	10
0	16	462	393	46	46	3·39	11	49	47	3	4	3·59	—
	17	366	355	37	41	3·02	12	67	48	2	7	4·32	—
	18	381	325	33	25	2·66	13	54	81	3	11	5·19	—
	19	313	349	37	34	2·56	14	46	49	4	6	3·65	—
	20	389	313	40	32	2·69	15	46	42	3	2	3·24	—
		Geometric mean:				2·85		Geometric mean:				3·94	14
−75	6	323	344	38	47	2·62	1	38	41	10	4	3·24	—
	7	358	388	38	39	2·87	2	108	104	8	9	8·0	—
	8	436	445	52	46	3·42	3	43	41	4	8	3·35	—
	9	401	389	39	38	3·02	4	41	57	5	3	3·79	—
	10	362	343	35	42	2·69	5	70	72	3	2	5·12	—
		Geometric mean:				2·91		Geometric mean:				4·42	16

between different dilutions of the same sample. Agreement between replicate samples was good in the control series but less good among irradiated samples. For instance, of the samples irradiated at 18°, the highest count was four times the lowest (see also Table 3). Although the cause is not known, this kind of variation has often been observed between similarly irradiated samples, as for instance in the results of Denny *et al.* (1959) for *Cl. botulinum* in phosphate buffer.

It has been shown by Riemann (1959) that the plating medium exerts a large influence on the counts of PA 3679 spores after irradiation in meat. In our experiments only one medium was used, so we have no evidence whether recovery of the irradiated spores was optimal.

Irradiations with 2 MeV electrons

The results in Table 1 indicate that after irradiation of spore suspension A with 1 Mrad survivals were 10, 14 and 16% for temperatures of 18°, 0° and −75° respectively. Analyses of variance, carried out so as to differentiate the effect of temperature between 18° and 0° from that between 0° and −75°, revealed that at the 5% level only the former difference was significant.

Table 2. *The effect of temperature on the survival of* Cl. botulinum *spores in minced pork irradiated with* 1 *Mrad of* 2 *MeV electrons*

(Data from expt 3, spore suspension B)

Irradiation temperature (°C)	Trial no.	Mean count (10^3/g)		Survivors (% of control)
		Control	Irradiated	
0	1	287	48·8	—
	2	301	16·4	—
	3	186	21·4	—
	4	145	26·3	—
	5	216	37·2	—
	Geometric mean	219	27·9	12·7
−75	1	247	44·2	—
	2	—	24·8	—
	3	194	32·1	—
	4	194	38·7	—
	5	183	19·8	—
	Geometric mean	203	30·6	15·1

Because any difference related to the act of freezing should be evident between 0° and −75°, this comparison was repeated in a further trial with electrons (experiment 3) at the same dose of 1 Mrad but using a different spore suspension (B). The results, summarized in Table 2, were similar to those of experiment 1 and again failed to show any significant difference between 0° and −75°.

Irradiations with gamma rays

More extensive results were obtained with gamma rays, of which a range of doses was given to samples inoculated with spore suspension B and held at 0° and −75°. The data are shown in Table 3.

Table 3. *The effect of temperature and dose on the survival of* Cl. botulinum *spores in minced pork irradiated with gamma rays*

(Data obtained with spore suspension B)

Irradiation dose (Mrad)	Viable count/g of pork in					
	Experiment 4		Experiment 5		Experiment 6	
	0°	−75°	0°	−75°	0°	−75°
None (Control)	$1{\cdot}33 \times 10^{5}$	$1{\cdot}84 \times 10^{5}$	$1{\cdot}14 \times 10^{5}$	$8{\cdot}54 \times 10^{4}$	$3{\cdot}34 \times 10^{5}$	$4{\cdot}49 \times 10^{5}$
	$6{\cdot}94 \times 10^{4}$	$1{\cdot}31 \times 10^{5}$	$5{\cdot}47 \times 10^{4}$	$8{\cdot}64 \times 10^{4}$	$3{\cdot}45 \times 10^{5}$	$3{\cdot}45 \times 10^{5}$
	$1{\cdot}04 \times 10^{5}$	$8{\cdot}15 \times 10^{4}$	$5{\cdot}09 \times 10^{4}$	$9{\cdot}20 \times 10^{4}$	$2{\cdot}61 \times 10^{5}$	$2{\cdot}34 \times 10^{5}$
	$6{\cdot}90 \times 10^{4}$	$6{\cdot}87 \times 10^{4}$	—	—	—	—
	$1{\cdot}09 \times 10^{5}$	$5{\cdot}80 \times 10^{4}$	—	—	—	—
1·0	$1{\cdot}00 \times 10^{4}$	$3{\cdot}47 \times 10^{4}$	$1{\cdot}17 \times 10^{4}$	$2{\cdot}24 \times 10^{4}$	$1{\cdot}41 \times 10^{4}$	$5{\cdot}41 \times 10^{4}$
	$7{\cdot}25 \times 10^{3}$	$3{\cdot}45 \times 10^{4}$	$1{\cdot}67 \times 10^{4}$	$2{\cdot}28 \times 10^{4}$	$1{\cdot}47 \times 10^{4}$	$3{\cdot}45 \times 10^{4}$
	$7{\cdot}25 \times 10^{3}$	$2{\cdot}95 \times 10^{4}$	$2{\cdot}06 \times 10^{4}$	$2{\cdot}02 \times 10^{4}$	$7{\cdot}07 \times 10^{3}$	$2{\cdot}42 \times 10^{4}$
	$6{\cdot}62 \times 10^{3}$	—	$5{\cdot}89 \times 10^{3}$	$1{\cdot}60 \times 10^{4}$	$1{\cdot}50 \times 10^{4}$	$2{\cdot}15 \times 10^{4}$
	$1{\cdot}12 \times 10^{4}$	$1{\cdot}40 \times 10^{4}$	—	—	—	—
1·3	—		436	$1{\cdot}01 \times 10^{4}$	—	
	—		—	$1{\cdot}28 \times 10^{4}$	—	
	—		943	$1{\cdot}28 \times 10^{4}$	—	
	—		767	$4{\cdot}60 \times 10^{3}$	—	
1·7	—		129	898	418	1810
	—		27·9	484	86·4	833
	—		38·3	432	129	1600
	—		195	784	143	634
2·0	—		—		13·9	80
	—		—		10·5	98
	—		—		17·4	91
	—		—		17·4	42

Table 4. *A summary of the effects of the irradiation temperature on the percentage of* Cl. botulinum *spores surviving a dose of 1 Mrad in minced pork*

Experiment and spore suspension		Survival (%) after irradiation with			
		Electrons		Gamma rays	
		0°	−75°	0°	−75°
1	A	14	16	—	—
2	A	18	—	—	—
3	B	13	15	—	—
4	B	—	—	11	35
5	B	—	—	18	23
6	B*	—	—	4	9·5

* Spore suspension B concentrated fourfold.

The effects of 1 Mrad will be considered first, and Table 4 shows the mean percentage survivals obtained after 1 Mrad at 0° and −75° in both the electron and gamma rays experiments. In experiment 4 the survival at 0° (11%) agreed well with the values obtained in the electron experiments, but the survival at −75° was distinctly higher (35%). Because there was satisfactory agreement between replicates, fewer were required at each dose in experiments 5 and 6 and a greater range of doses was used. In these two experiments further comparisons between the survival at 0°

and −75° after a dose of 1 Mrad of gamma rays were obtained, and whereas experiment 5 gave results similar to those with electrons, revealing no significant effect of temperature, experiment 6 resembled experiment 4 in that there were more than twice as many survivors at −75° as at 0°, though the degrees of inactivation were much higher generally than in all the earlier experiments. This last difference is remarkable, because the same spore suspension (B) has been used in experiments 3–6, although it was more concentrated in experiment 6.

The data for all the doses used in experiments 5 and 6 are shown in Fig. 1. Despite the larger inoculum and greater inactivation at 1 Mrad in experiment 6, the responses to higher doses were very similar in the two experiments. It is evident that the exponential relation between survivors and dose held only at doses above about 1 Mrad, as others have observed previously. Fig. 1 also shows that experiment 5 covered a smaller mortality range than experiment 6 and less of the exponential phase: hence the use of a larger inoculum and a higher dose in experiment 6.

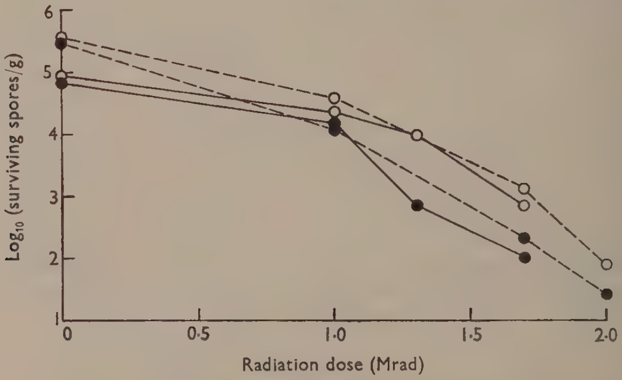

Fig. 1. The effect of temperature and dose on the inactivation by gamma rays of spores of *Cl. botulinum* suspended in minced pork packed under nitrogen. Full circles, 0°; open circles, −75°. Continuous lines, expt 5; broken lines, expt 6.

The inactivation by gamma rays in frozen samples was very similar to that observed by Denny *et al.* (1959) and Pratt *et al.* (1959) for *Cl. botulinum* spores in frozen pork. The comparison between the two temperatures is also in general agreement with Denny's results for spores in phosphate buffer, irradiated frozen and at room temperature, although the difference found in their experiments was rather greater than in ours.

Discussion

It is generally agreed that the relationship between the logarithm of the survival of irradiated *Cl. botulinum* spores and dose becomes linear only at doses above about 1 Mrad, while at lower doses the exponent increases with dose (see Rayman & Byrne, 1957). This in fact happened in our experiments, as Fig. 1 shows. The calculation of the dose necessary for the survival to reach 10^{-12} of the spores initially present depends therefore on the assumption that the death rate, observed experimentally at doses from 1–2 Mrad, remains constant down to the survival level of 10^{-12}.

From our experiments, the relationship between irradiation at −75° and room temperature has to be inferred from two direct comparisons, between −75° and 0° and between 0° and 18°, and these will be discussed separately.

In the first analysis, we took observations from all three gamma ray experiments, at doses from 1–2 Mrad, calculated the fraction inactivated by each dose at the two different temperatures, and then used the mean values for deriving the regression lines for each temperature. These indicated 'sterilizing' doses (i.e. those giving a survival of 10^{-12}) of 4·3 and 4·7 Mrad respectively (Ingram *et al.* 1959). This procedure did not, however, seem wholly satisfactory, since experiment 4 contributed observations at 1 Mrad only (Table 3), and as it happened that the survivals at −75° were comparatively high (Table 4), the −75° average at this dose was raised without corresponding effect at the higher doses: also, the data for these irradiated samples were imperfect (Table 3). It therefore seemed better to omit this experiment. Furthermore, in the two remaining experiments, the degrees of inactivation for a given dose were rather different, and this introduced a large variance when they were combined, as an analysis of variance computed on degree of inactivation showed. Hence, the actual counts for these two experiments were taken separately (*cf.* Fig. 1): separate regression lines were calculated, and the corresponding doses found for 10^{-12} survivors of the initial count. The values so derived are given in Table 5, from which it is clear that, although both experiments indicate a higher sterilizing dose at −75° than at 0°, the differences were small and probably not significant. Further doubt appears on scrutiny of Fig. 1, for it seems that, at least at −75°, the exponential part of the curve may have been reached only with doses above 1·3 Mrad, which would mean that the estimates of the sterilizing dose for that temperature are too high.

Table 5. *Sterilizing* (10^{-12} *survival*) *doses calculated* from the regressions of log survivors on dose‡ for* Cl. botulinum *spores irradiated in minced pork at* 0° *and* −75°

Experiment and irradiation temperature		Regression constants†		Calculated sterilizing dose† (Mrad)		
		α	β	Maximum likelihood	Lower limit	Upper limit
5	0°	7·02 ± 0·34	−3·02 ± 1·17	4·69	3·29	12·39
	−75°	8·60 ± 0·33	−2·18 ± 0·95	6·27	4·26	11·39
6	0°	7·02 ± 0·35	−2·87 ± 0·50	4·71	3·90	5·86
	−75°	7·14 ± 0·36	−2·53 ± 0·86	5·37	3·90	8·36

* A regression line $y = \alpha + \beta x$ (where y is log. no. of bacteria/g, x is the radiation dose in Mrads and β is the negative reciprocal of the decimal reduction dose, D, in Mrads) was fitted to the observations in experiments 5 and 6 (cf. Fig. 1), and its 95% confidence limits were computed. For each experiment and temperature the 10^{-12} level of survival (compared with the control mean) was determined. The line corresponding to 10^{-12} survival then cut the regression line at a value of x equivalent to the sterilizing dose (maximum likelihood). The 95% confidence limits of this dose included the range of values of x which could be obtained by varying α and β within their 95% confidence limits.

‡ Doses of 1 Mrad and over. † With 95% confidence limits.

Comparison between 18° and 0° is made on the basis of experiment 1 (Table 1), which gave mean survivals of 10% and 14% respectively after a 1 Mrad dose of electrons. This comparison between 0° and 18° is uncertain for several reasons. First, as already explained, it had to be confined to doses not exceeding 1 Mrad. Second, the indication that there was a significant difference between 0° and 18°, but not between 0° and −75°, is counter to the view that such differences relate to the act of freezing just below 0° (Wood, 1954). Third, for our comparison, the different survivals actually observed have to be converted into comparative doses on the basis of an assumed survivor-dose relationship, and this transformation can be only approximate, since the precise relation appears to have varied from one experiment to another (see Fig. 1). Nevertheless, the indication that any effect of temperature in the range 0° to 18° is small is similar to that obtained by other workers, and the uncertainties here are trifling compared with those involved in the long extrapolation to survival levels of 10^{-12}.

The computed relationships obviously depend to an altogether undesirable degree on assumptions about the basis of computation. Further, according to Riemann (1959), a different plating medium might have given different results.

The general inference to be drawn from our experiments is that low temperature has little effect on the sterilizing dose of ionizing radiations; but it has become apparent that, because of limitations inherent in this method of approach, such experiments would be incapable of establishing appreciable differences in the theoretical sterilizing dose at different temperatures, if any existed. The central difficulty is to demonstrate the existence of an exponential relationship over a sufficient part of the dose-mortality curve, in which respect our data resemble those obtained by other workers in similar experiments. To use larger initial numbers of spores should be advantageous, but there are the two difficulties of getting very dense suspensions of viable *Clostridium* spores (Halvorson, 1957) and of adding more than a small proportion of spore suspension to meat without so changing the composition of the meat as to make it unrepresentative. The lower end of the curve is limited by the quantity of material that can be examined in a plate count, and to extend it appreciably requires the examination of whole containers, preferably on a positive-negative basis as in dilution counts to avoid plating difficulties. Here, however, large numbers of containers are needed to get statistically significant results and this leads at once to procedures such as those advocated by Schmidt & Nank (1960), which give the decimal reduction dose (D-value) at high doses, and the best guarantee of the validity of the assumption that destruction is approximately exponential with dose lies in the broad agreement of such values with those indicated from data like ours.

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THE EFFECT OF CHLORTETRACYCLINE ON EVISCERATED TURKEYS COMMERCIALLY PROCESSED IN GREAT BRITAIN

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SUMMARY: Ten turkeys, weighing approximately 10 lb, of known history were processed, bisected, randomly distributed between two groups and cooled for 4 hr in water and ice. One group was treated with 10 p/m of chlortetracycline, then all the half carcases were stored at 3°. The treatment with chlortetracycline significantly delayed the onset of spoilage. Bacterial counts of 10^6 organisms/cm^2 of skin were obtained with the control halves on the 7th day and on the treated halves on the 14th day. The development of 'off' odours was delayed from 10·1 to 22·2 days. In a second experiment twenty 10 lb turkeys from a similar source and of similar history were processed and treated as described above. Immediately after treatment they were frozen for 19 weeks, thawed, and stored at 3°. These were not examined microbiologically but sensory examination showed that the treatment with chlortetracycline extended the storage life from 7·7 to 10·3 days.

The effect of the antibiotic chlortetracycline upon the keeping quality of chilled and frozen chickens has been reviewed recently by Walker, Taylor & Davis (1960) and by Walker & Taylor (1960), who confirmed the efficacy of the application under commercial conditions characteristic of poultry processing operations in Great Britain. It has been reported that the application of 10 p/m of chlortetracycline to eviscerated turkeys, which subsequently were chilled or deep frozen, prolonged their storage life at 1·7° by 7–10 days (Windlan *et al.* 1956; Kraft, Elliott & Brant, 1958).

Data obtained from a survey of the table poultry processing industry (Taylor & Walker, 1959) indicated that a similar treatment might be feasible for this type of poultry processed by some of the methods in use. The purpose of these experiments, therefore, was to evaluate the application of chlortetracycline to eviscerated turkeys which, after treatment, were stored at chilled temperatures only, or alternatively, were deep frozen for 4 months, thawed, and then held in chilled storage. The investigations were carried out on separate occasions in one commercial station which normally processed over 50,000 birds a year, and in which the methods of processing and standard of hygiene could be related to the normal practice of certain types of turkey packing stations in Great Britain.

Materials and Methods

Processing and grouping of birds

'Chilled' birds. Ten broad breasted, hybrid white hen turkeys, intensively reared and weighing approximately 10 lb, were distributed to one packing station operating

under the 'good commercial' conditions described by Walker, Taylor & Davis (1960). The birds received a commercial turkey ration which contained oxytetracycline at a level of 100 g/ton for the first 2 weeks of life, and thereafter at 10 g/ton. During the normal processing operations of the station they were stunned, bled via the jugular vein for 1 min, scalded at 50° for 2 min, mechanically plucked, hand stubbed, eviscerated, left and right sides labelled, bisected and rinsed in water. The half carcases were distributed at random between two groups so as to provide an equal number of unmatched left and right halves in each group. Four additional carcases were similarly treated and used for temperature measurements as described by Walker & Taylor (1960). Giblets were not included.

'Frozen' birds. On a second occasion 20 turkeys were used. The source, breed, nature of rearing, and place and method of processing were similar in all respects to those described above.

Treatment with chlortetracycline

'Chilled' birds. Each group of 10 half carcases was submerged for 4 hr in a tank containing a mixture of water and ice. One tank contained 10 p/m of chlortetracycline as described by Walker & Taylor (1960). The halves were removed from their respective tanks, momentarily drained, enclosed individually in Cryovac (Dewey and Almy Chemical Co.) bags, packed with a small quantity of ice in an insulated container, transported to the laboratory where each was transferred from the Cryovac bag to a sterilized cellophane box and stored at 3±1°.

'Frozen' birds. Each group of 20 half carcases was chilled, as described above, and one group was treated with 10 p/m of chlortetracycline. These were similarly drained and wrapped, but were then blast frozen at –29° for 19 hr, stored under identical conditions at –18° for 19 weeks, transported in an insulated vehicle to the laboratory and stored at 3±1°. The carcases thawed to an internal body temperature of 1° within 30–36 hr; this stage was taken as the start of the chilled storage period. The halves were then removed from the bags, transferred to separate cellophane boxes, and storage at 3±1° continued.

Microbiological and sensory examination

'Chilled' birds. The nature and frequency of the microbiological and sensory examinations of the carcases were similar in all respects to those described by Walker, Taylor & Davis (1960), but the examinations continued until the 27th day.

'Frozen' birds. The sensory examination, as described above, was carried out on the carcases. They were not examined microbiologically.

RESULTS

Microbiological examination of 'chilled' carcases

Comparisons between the control and treated half carcases were not possible after 21 days of storage as most of the controls had then been discarded due to their offensive condition. Statistical analyses were carried out as described by Walker, Taylor & Davis (1960). Table 1 shows the effect of the treatment with 10 p/m of chlortetracycline upon the development of bacteria and yeasts during storage at 3°.

Table 1. *The effect of chlortetracycline (CTC) treatment on the bacterial and yeast populations of turkey carcases* stored at 33° after processing*

Days of storage at 3°	CTC concn. (p/m)	Colony counts*/cm² of skin (a) and corresponding significance ratios† (b) for					
		Bacteria on Yeastrel milk agar after				Yeasts on glucose yeast agar after 6 days at 18°	
		4 days at 18°		14 days at 5°			
		(a)	(b)	(a)	(b)	(a)	(b)
1	0	9.6×10^{4}	3·9	3.0×10^{3}	4·8	1.0×10^{3}	8·1
	10	1.1×10^{5}		2.0×10^{3}		1.0×10^{3}	
3	0	1.4×10^{5}	5·3	‡		‡	
	10	4.6×10^{4}					
7	0	1.5×10^{6}	7·6	4.3×10^{6}	29·0	1.0×10^{4}	5·6
	10	5.0×10^{4}		5.0×10^{4}		2.0×10^{4}	
10	0	1.2×10^{8}	26·7	‡		‡	
	10	6.0×10^{5}					
14	0	3.2×10^{9}	7·6	7.2×10^{9}	23·3	5.3×10^{6}	68·2
	10	7.1×10^{6}		3.3×10^{6}		2.9×10^{7}	
17	0	1.9×10^{9}	2·7	‡		‡	
	10	2.0×10^{7}					
21	0	7.4×10^{9}	12·1	1.2×10^{10}	21·4	7.6×10^{6}	14·6
	10	1.1×10^{8}		7.1×10^{7}		2.0×10^{8}	
24	0	§		‡		‡	
	10	5.6×10^{7}					
27	0	§		§		§	
	10	1.7×10^{8}		3.9×10^{8}		2.4×10^{8}	

* Geometric mean of experiments each with 10 half carcases at each level of treatment, all carcases being examined on each occasion.

† Significance ratio. When the ratio of the larger number to the smaller exceeds the significance ratio, the difference between the two values is significant ($P<0.001$).

‡ Not measured. § Carcases discarded due to offensive condition.

The proliferation of bacterial colonies capable of growth at 5° and at 18° was delayed appreciably by 10 p/m of chlortetracycline, the differences between the groups being highly significant ($P<0.001$) by the 7th day, when the counts on the control birds were of the order of 10^6/cm² of skin. Similar proportions were not reached on the treated halves until the 14th day. During storage, the yeast populations increased on all the birds, although the rate of increase was greater in the treated group after the 7th day. Significant differences ($P<0.001$) between the control and treated halves were not apparent until after the 14th day. Notable changes in the mould populations were not apparent until after the 21st day. At the time of the disposal of the control halves, the counts reached 10^2–10^4/cm² of skin, and at the 27th day the mould counts on the treated halves varied between 10^3 and 10^6/cm² of skin.

Sensory examination of 'chilled' carcases

Appearance. The mean times at which microbial growth was first visible on the half carcases are shown in Table 2.

Table 2. *The effect of chlortetracycline (CTC) on the development of visible microbial growth on turkey carcases stored at 3° after being processed at different temperatures*

CTC concn. (p/m)	Data for carcases processed at					
	3°			−18°		
	Mean storage life* (days)	S.D.†	Mean extension of life (days)	Mean storage life* (days)	S.D.‡	Mean extension of life (days)
0	10·1	2·6	7·5	7·2	1·4	2·2
10	17·6	1·3		9·4	1·3	

* Based on absence of visible microbial growth. † Standard deviation of means for 10 half carcases. ‡ Standard deviation of means for 20 half carcases.

Bacterial and yeast colonies, which could not be differentiated visually, first appeared on the pleural and peritoneal membranes and later extended over the entire visceral cavity and the external skin. Treatment with chlortetracycline delayed the rapid development of colonies and prevented the formation of ‘bacterial

Table 3. *The effect of chlortetracycline (CTC) on the development of odours on turkey carcases stored at 3° after being processed at different temperatures*

Duration of storage at 3° (days)	CTC concn. (p/m)	Data for carcases processed at			
		3°		−18°	
		Mean odour assessment*	S.D.†	Mean odour assessment*	S.D.‡
3	0	1·1	0·1	1·5	0·2
	10	1·2	0·2	1·4	0·2
6	0	1·5	0·4	2·1	0·3
	10	1·3	0·3	2·0	0·3
8	0	2·2	0·6	3·4	1·0
	10	2·0	0·2	2·3	0·1
10	0	3·2	0·7	5·3	0·8
	10	2·0	0·0	2·7	0·2
13	0	4·2	1·0	6·6	0·3
	10	2·3	0·3	4·5	0·5
15	0	5·0	1·1	7·0	0·0
	10	2·3	0·3	5·8	0·3
17	0	5·7	1·0	§	0·3
	10	2·5	0·3	6·5	
20	0	6·5	0·9	§	0·1
	10	2·6	0·6	6·9	
22	0	6·7	1·6	§	0·0
	10	3·0	1·0	7·0	
24	0	7·0	0·0	§	
	10	3·6	1·2	§	
27	0	§	§	§	
	10	4·6	1·1	§	

* Odour assessment: 1·0–3·0, fresh; 3·0–5·0, faint but distinctive ‘off’ odour; >5·0, strong and offensive ‘off’ odour (Walker, Taylor & Davis, 1960).

† Standard deviations of means for 10 half carcases. ‡ Standard deviations of means for 20 half carcases. § Carcases discarded due to offensive condition.

slime'. Generally, faint mould growth started to appear on the treated carcases at the time when 'off' odours were apparent.

Odour. The mean odour scores of the groups of carcases are shown in Table 3, and the mean durations of storage prior to the development of a distinctive 'off' odour (score 5) are given in Table 4.

Table 4. *The effect of chlortetracycline (CTC) on the duration of storage, as assessed by odour, of turkey carcases stored at 3°*

Odour score*		No. of half carcases examined	Mean storage life at 3° days with CTC concn. (p/m) of:		S.E.†	Extension of storage life due to CTC (days)
			0	10		
Fresh	≯3	10‡	10·1	22·1	±1·3	12·0
		20§	7·7	10·3	±0·2	2·6
Faint 'off' odours	≯4	10‡	13·5	25·1	±1·8	11·6
		20§	8·6	12·3	±0·3	3·7

* See Walker, Taylor & Davis (1960). † Standard errors of differences in means. ‡ Processed at 3°. § Processed at −18° and subsequently thawed to 3°.

Treatment with 10 p/m of chlortetracycline delayed the development of an 'off' odour from 10·1 days, when the mean count on the control halves had reached $1{\cdot}2\times10^{8}$ organisms/cm^{2} of skin, to 22·1 days, when the bacterial and yeast counts were of the order of 10^{8} and 10^{7}/cm^{2} of skin, respectively. This represented an increase in storage life of 118·8%. The difference between the control and treated groups was highly significant ($P<0{\cdot}001$).

Sensory examination of 'frozen' carcases

Appearance. The mean times at which microbial growth was first visible on the half carcases are shown in Table 2. The location of colonies was similar to that in the 'chilled' carcases. Although treatment with chlortetracycline delayed the rapid development of colonies, the rate of increase was notably greater than in the experiment where the carcases were not 'frozen'.

Odour. The mean odour scores of the half carcases are shown in Table 3, and the mean duration of storage prior to the development of a distinctive 'off' odour are given in Table 4. Treatment with chlortetracycline delayed the development of a faint 'off' odour from 7·7 to 10·3 days. Thus the mean storage period was increased by 33·8%. Again the difference between the groups was highly significant ($P<0{\cdot}001$).

DISCUSSION

The results obtained for turkeys which were only subjected to chilled storage after processing were in agreement with those reported by other workers in that the application of 10 p/m of chlortetracycline approximately doubled the storage life. The effect of chlortetracycline on the birds which were deep frozen after storage was similar to that obtained in the previous experiment on deep frozen chickens (Walker & Taylor, 1960).

Direct comparisons between the two experiments cannot be made, as the birds were processed on separate occasions and because the nature and size of the initial bacterial populations on the frozen carcases were not determined. Nevertheless, between the 'chilled' and 'frozen' carcases which were treated with chlortetracycline, there was an appreciable difference in the duration of storage prior to the development of an 'off' odour, suggesting that the activity of enzymes of psychrophilic bacteria cannot be disregarded. Peterson & Gunderson (1960), in discussing this problem, which arises with frozen foods during the process of thawing, pointed out that 'off' odours may become apparent before the psychrophilic bacteria reach the proportions which are normally associated with spoilage. This indicates the significance of enzyme activity in this respect.

Windlan *et al.* (1956) found that the period of dipping had a significant effect upon the subsequent storage life of turkeys treated with chlortetracycline prior to deep freezing. Therefore, an increase in the period of dipping might bring about an extension greater than that obtained in this experiment.

The authors wish to express their thanks to the Directors and Staff of the Poultry Processing Station which co-operated in the experiments; to the staff of the Meat Section of the National College of Food Technology for participating in the sensory examinations; and to Mr. M. H. Quenouille for the statistical analysis of the results.

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THE SANITATION OF FISH BOXES. III. A COMPARISON OF PLASTIC COATED AND UNCOATED WOODEN BOXES

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SUMMARY: In comparisons of the extents to which new plastic coated and uncoated commercial wooden fish boxes became contaminated when filled with fish, and of the efficiencies of various methods of cleaning them, it was established that the coated boxes were less contaminated before cleaning and were generally cleaned more efficiently by the various methods examined. Scrubbing was essential for uncoated boxes, but hosing with cold water was reasonably satisfactory for coated boxes. The sanitizers and detergents in the concentrations used did not materially enhance the cleaning effect of water alone. There was some evidence that during the experiment a build up of bacteria occurred on coated boxes cleaned by methods which did not involve scrubbing or hosing with a solution of a quaternary ammonium compound.

It has been shown (Spencer, 1959, 1960) that commercial wooden fish boxes cannot be cleaned satisfactorily with cold water alone, either by hosing or by scrubbing, and that although the use of relatively strong sanitizers such as solutions containing 1,000 p/m of available chlorine or 1% of various quaternary ammonium compounds reduced the viable bacterial count by 90–99%, the residual contamination still amounted to millions/cm^2 of box surface. In this work certain of the boxes had, when new, been coated with a urea-formaldehyde resin to render them impervious to the bacteria laden moisture and slime, and to provide a surface which would be more easily cleaned than that of the normal wooden box. This resin had been painted on and the coating, when hard, was quite resistant to cold water, though the use of steam for sterilizing purposes was not recommended. The coating was also resistant to organic detergents and solvents, but not to strongly acidic or alkaline detergents. These boxes were indeed much more easily cleaned than normal ones, and also had a much lower initial count, and in view of their apparent advantages further experiments were carried out to substantiate and extend these findings. It was also planned to discover which of several methods of cleaning would give the best results, and whether there was any build up of bacteria on either type of box. The results of these experiments are reported below.

Materials and Methods

Contamination, cleaning and examination. Twelve new plastic coated boxes and 12 new uncoated boxes were put through four cycles of contamination and cleaning. The boxes were contaminated by being filled with fish from the market and allowed to stand full for several hours, in some cases overnight. After being emptied they were allowed to stand for a further 1–2 hr, when samples were taken for viable counts. The boxes were then cleaned, allowed to drain in an upright position for 10 min and sampled again. Several days later this cycle was repeated. The samples were obtained by the scraping method previously described (Spencer, 1959), and care was taken throughout that no two samples were obtained from the same area of a box. For the viable counts, duplicate roll tubes and a sea water Lemco agar medium were used (Spencer, 1961). Incubation was at 20° for 5 days.

Methods of cleaning. Pairs of the two types of box were used for each method of cleaning, and the mechanical methods of washing were as previously described (Spencer, 1960).

Six methods of cleaning were used, namely: hosing with cold water, the usual commercial method; scrubbing with cold water; hosing with a nonionic detergent in 0·1% concentration (sanitizer B, Spencer, 1960); hosing with a 1% solution of a caustic detergent not previously used in these investigations and chosen as a representative of a type with strong dissolving powers; hosing with cold water alone followed by hosing with a sodium hypochlorite solution containing 100 p/m of available chlorine (sanitizer A, Spencer, 1960); and hosing with cold water alone followed by hosing with an 0·1% solution of the quaternary ammonium compound, cetyltrimethylammonium bromide (sanitizer D, Spencer, 1960).

Regarding the use of hypochlorite, it is true that 100 p/m of available chlorine was shown in previous investigations to have no marked bactericidal effect with old wooden boxes, but it was considered that it might prove effective with new boxes, which would presumably have a lower level of contamination. Also, cetyltrimethylammonium bromide, which had been found to have considerable effect even with old wooden boxes, might be expected to be more efficient on the less contaminated new boxes.

RESULTS

The levels of bacterial contamination on both types of box over the four cycles of operation, before and after cleaning, and for all methods of cleaning combined, are shown in Table 1 as the proportions of counts falling within certain ranges. As judged by the median values, the level of contamination of the uncleaned uncoated boxes was more than 50 times that of the uncleaned coated boxes, and this difference was even greater after cleaning, where the level for the cleaned coated boxes was only one threehundredth of that on the cleaned uncoated boxes. The general effect of cleaning was also greater with the coated boxes, where the reduction was to one fiftieth, as compared with a reduction to one tenth with the uncoated boxes.

The efficiencies of the various methods of cleaning, as measured by the percentage reductions in viable count, are shown in Table 2. On occasions the count after cleaning was greater than that before cleaning. This situation occurred mainly with the coated boxes, which were dry when sampled before cleaning, probably because this dryness would have led to a lower initial count, that is to an apparently cleaner box, and consequently to a much reduced cleaning effect. Such anomalous results were not used in the calculations of the mean percentage reductions. In one experiment box 20, which was normally cleaned with the caustic detergent, was inadvertently cleaned by hosing with water followed by hosing with 100 p/m chlorine solution.

while the reverse happened with box 22. In Table 2 the percentage reductions in these two cases are given against the appropriate cleaning method. Box 22 was lost during the contamination stage of the third cycle.

Table 1. *The effect of cleaning* on the incidence of different levels of bacterial contamination on uncoated and coated wooden fish boxes*

Range of bacterial counts/cm² of surface sampled	Percentages of samples with counts in ranges indicated, on boxes			
	Uncoated		Coated	
	A‡	B‡	A‡	B‡
$<10^1$	0	0	0	21
10^1–10^2	0	2	4	23
10^2–10^3	0	9	19	19
10^3–10^4	0	13	21	17
10^4–10^5	13	28	42	8
10^5–10^6	42	42	10	2
10^6–10^7	37	6	4	0
10^7–10^8	8	0	0	0
$>10^8$	0	0	0	0
No. of samples	46	46	48	48
Extreme counts	$1{\cdot}3\times10^4$– $16{\cdot}5\times10^6$	$1{\cdot}1\times10^2$– $2{\cdot}5\times10^6$	$<3\times10^1$– $8{\cdot}3\times10^6$	$<3\times10^0$– $5{\cdot}2\times10^5$
Median values	$9{\cdot}2\times10^5$	$7{\cdot}6\times10^4$	$1{\cdot}4\times10^4$	$2{\cdot}3\times10^2$

* Data for all six methods of cleaning have been combined.

‡ A, before cleaning; B, after cleaning.

From Table 2 it can be seen that scrubbing was the best method for cleaning both types of box. This was especially so for the uncoated boxes, as it was the only method which gave a constant reduction in viable count approaching 99%. Hosing with water alone was reasonably efficient with the coated boxes, giving reductions of over 90%, but was comparatively inefficient with the uncoated boxes, although it did give marked reductions in two cases. The use of detergents resulted in no marked improvements over water alone, with either type of box, and the two disinfectants, in the concentrations used, did not materially enhance the effect of water alone on either type of box.

The boxes used in this investigation were initially new and were used, that is contaminated and washed, only four times, whereas in commercial practice boxes are used very many times. There would thus be the possibility in practice, where washing may be inefficient, of a progressive increase in the residual contamination in the boxes after washing. It is consequently possible that although coated boxes appear to be superior to uncoated boxes in respect of degree of contamination and ease of cleaning when new, this superiority may be lost on continued use because of the development of a high residual contamination. Fig. 1 shows the levels of residual contamination on coated and uncoated boxes after each washing. It is clear that although there was no evidence of a build up of bacterial contamination on the uncoated boxes within the four cycles, this was occurring in those coated boxes which were not scrubbed or washed with the QAC solution.

Table 2. *The effect of six methods of cleaning on the bacterial load on coated and uncoated wooden fish boxes*

No. of box	and method of cleaning	Reduction (%) in load at cleaning cycle 1	2	3	4	Mean reduction in load (%)
Coated boxes						
1	Hosed, water	99·9	>90·0	95·6	94·6	>96·5
2		99·9	99·12	93·1	99·6	
3	Scrubbed, water	*	*	99·3	99·8	99·7
4		*	*	99·99	99·6	
5	Hosed, nonionic detergent	99·7	>96·7	99·3	*	>98·7
6		99·96	99·97	96·5	*	
7	Hosed, caustic detergent	99·93	82·0	*	*	>93·7
8		99·99	>96·7	90·0	*	
9	Hosed, water; hosed, 100 p/m chlorine	82·0	99·1	54·0	71·0	81·2
10		99·3	*	*	22·0	
11	Hosed, water; hosed, 0·1% QAC	*	90·0	87·0	99·0	>96·5
12		99·8	99·5	>99·96	99·98	
Uncoated boxes						
13	Hosed, water	95·0	65·0	96·9	99·9	80·0
14		20·0	78·5	85·0	99·98	
15	Scrubbed, water	99·97	>99·8	99·9	99·96	>99·1
16		99·98	98·8	95·7	98·95	
17	Hosed, nonionic detergent	*	91·4	92·0	87·0	68·9
18		65·0	44·0	85·0	86·5	
19	Hosed, caustic detergent	83·0	92·5	99·6	86·0	82·2
20		97·9	32·0†	59·0	97·4	
21	Hosed, water; hosed, 100 p/m chlorine	90·1	81·6	*	97·4	90·4
22		*	92·4†	‡	‡	
23	Hosed, water; hosed, 0·1% QAC	96·5	49·8	73·5	76·0	78·0
24		36·0	99·97	97·8	99·99	

* Count after cleaning higher than count before cleaning. † Boxes wrongly cleaned, see text

‡ Box 22 was lost after the second cleaning.

DISCUSSION

It has been shown that under comparable conditions of contamination plastic coated wooden fish boxes became much less highly contaminated than uncoated wooden boxes, and that allied with this lower initial contamination was a greater degree of cleanability. Of the methods of cleaning investigated, only scrubbing could be considered satisfactory for uncoated boxes, although this is frequently held to be impracticable in commercial practice. The use of appropriate sanitizers with hosing may effect a reduction in the bacterial count greater than 99% in certain cases, but would still leave behind the residual detritus and this method of cleaning has been shown to be much less effective with older uncoated boxes (Spencer, 1960). With the coated boxes scrubbing was also the most effective method of cleaning, but the use of a strong jet of water, allied with a suitable sanitizer, gave a 90–99% reduction or even better, which at first sight would appear satisfactory. However, the evidence for a build up on coated boxes with continued use and unsatisfactory cleaning indicates that the advantages they offer initially may well be lost if they are not cleaned efficiently, preferably by scrubbing.

Although there was no evidence of a build up of residual contamination after washing the relatively new uncoated boxes, it should be noted that the counts obtained both before and after washing them (Table 1) were considerably lower than those obtained by identical methods on older boxes (Spencer, 1959). It is probable that extended use, with inefficient washing, would lead to higher levels of residual contamination.

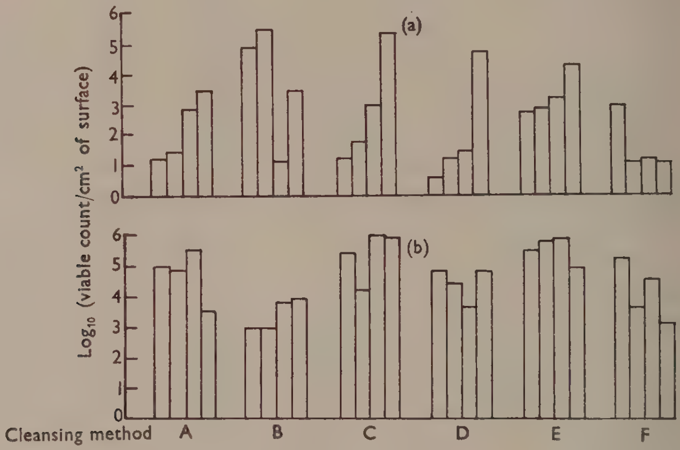

Fig. 1. The changes in the levels of residual bacterial contamination on (a) cleaned coated and (b) cleaned uncoated wooden fish boxes during four cycles of contamination and cleaning, with several cleaning methods.
Each column represents the mean value for a pair of boxes. Cleaning method:—A, hosed, water; B, scrubbed, water; C, hosed, nonionic detergent; D, hosed, caustic detergent; E, hosed, water; hosed, 100 p/m chlorine; F, hosed, water; hosed, 0·1% QAC.

As mentioned in previous reports (Spencer, 1959, 1960) the extent to which results obtained with fish boxes can be applied to other wooden surfaces, within and outside the fish industry, is not as yet known, although it is considered that the problems, particularly within the fish industry, are similar. Highly contaminated wooden surfaces on the trawler may lead to enhanced spoilage of fish in contact with them, or to the development of unusual types of spoilage (MacCallum, 1955), and contamination of the flesh of fish from wooden surfaces during the filleting operation may also enhance subsequent spoilage. The use of a suitable plastic coating would lead to reduced contamination and easier and more efficient cleaning of the surfaces concerned, with a reduction in their deleterious effect on fish in contact with them.

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THE LECITHINASE AND LIPASE ACTIVITIES OF MEMBERS OF THE GENUS *PSEUDOMONAS*

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AND

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SUMMARY: The group I pseudomonads of Shewan, Hobbs & Hodgkiss (1960) generally produced both lipase and lecithinase, the species *Pseudomonas aeruginosa* being consistently active. Strains belonging to the other three groups were comparatively inactive.

INTRODUCTION

THE ACTION of Gram-positive bacteria on egg yolk and other media containing lecithin and fat has been extensively studied. Lipid breakdown by Gram-negative bacteria has been determined in the main by the use of substrates such as margarine, butter and cotton seed oils including a pH indicator.

Starr & Burkholder (1942) studied a large number of plant pathogenic bacteria and found that certain organisms in the genus *Phytomonas* including the majority of xanthomonads studied were able to decompose fat as shown by the spirit blue-agar method, whereas only a small proportion of the phytopathogenic pseudomonads were lipolytic. Goldman & Rayman (1952) tested 8 strains of pseudomonads and found only one was inactive. In 1959 Rhodes examined 169 strains of *Pseudomonas fluorescens* and found that 123 produced opalescence on egg yolk agar; these strains also gave positive reactions on olive oil, margarine oil and tributyrin media, though some strains which gave negative results on egg yolk agar gave positive results on the other media.

A number of other authors have reported production of opalescence on egg yolk media by strains of *Pseudomonas* (Crook, 1942; Klinge & Gräf, 1959; Klinge, 1957; Willis, 1960).

Felsenfeld (1944) demonstrated lecithinase activity by *Vibrio comma* and the El Tor vibrio, and Willis & Gowland (1960) examined a limited number of pseudomonads and found that *Ps. aeruginosa* characteristically produced an opalescence in egg yolk agar which was probably due to both lecithinase and lipase activity.

In the present investigation a wide range of pseudomonads has been examined. All the strains examined were divided into four groups according to the determinative scheme of Shewan *et al.* (1960) and the lecithinase and lipase activities of these groups were determined.

MATERIALS AND METHODS

Cultures

A total of 129 strains was examined and the organisms were grouped according to the scheme of Shewan *et al.* (1960) as follows.

Group I. This group comprised cultures of *Ps. aeruginosa* and other green fluorescent pseudomonads. The former consisted of 60 strains isolated from human infections, 7 strains from the National Collection of Industrial Bacteria (NCIB 950, 5940, 6750, 6751, 8295, 8297, 8298) and 4 stock strains. The other green fluorescent pseudomonads comprised: NCIB 3756, 8026, 8178, 8194, 8249, 8286, 8248, 8376, 9046; 1 strain from the National Collection of Marine Bacteria (NCMB 406); 6 cultures from spoiling fish; 1 culture from a spoiling egg; and 1 stock strain each of *Ps. syringae*, *Ps. medicaginis*, *Ps. marginalis*, *Ps. schulkylliensis*, *Ps. putida* and *Ps. striata.*

Group II. This group consisted of NCIB 8176, 8296, 8542 and 3 strains from spoiling fish.

Group III. This group comprised: NCIB 8177 and 8226; NCMB 130 and 155; 6 strains from the Torry Research Station collection (A1006, A1010, A1030, A1034, A1052, A1074); and 11 strains from spoiling fish.

Group IV. Included in this group were 3 strains from the Torry Research Station collection (A1008, A1024, A1042) and 5 strains from spoiling fish.

Media

Egg yolk agar, cream agar and lecithin agar as described by Willis (1960) were used, except that Fildes extract of red cells was omitted. Nutrient agar was used as a control medium.

Inoculation and incubation of media

Each strain was inoculated on to all the media as a streak inoculation from an actively growing broth culture. Plates were inoculated so as to accommodate four strains each and were incubated for 48 hr at 37° or 3 days at 20° depending on the temperature requirements of individual strains.

Interpretation of results

Lecithinase and/or lipase activity were determined as described by Willis (1960).

RESULTS AND DISCUSSION

The results are summarized in Table 1. Of the organisms in group I, all strains of *Ps. aeruginosa* produced opalescence on all three media. This, along with the staining reaction, indicates that both lipase and lecithinase were produced. Seventeen of the other green fluorescent pseudomonads also gave these results, whereas 6 strains produced no reaction on any of the media (NCIB 8248, 8249; NCMB 406; *Ps. putida*, *Ps. striata* and one of the strains from fish).

Except for two strains in group II (one from fish and NCIB 8542) and one strain in group III (A1010), the remaining 32 strains produced no reactions. It is of interest to note that only one strain (in Group II) produced lipase but no lecithinase.

The apparently inconsistent results given by a strain in Group II and one in group III (see Table 1) can probably be explained in terms of the relative amounts of lipase produced during the standard incubation time of 3 days.

Comparison of these results with early work is sometimes difficult owing to changes in the definition of the genus *Pseudomonas*. The organisms referred to as *Phytomonas* spp. by Starr & Burkholder (1942) included xanthomonads as well as pseudomonads, but whether or not the latter produced green fluorescent diffusible pigments and broke down glucose in an oxidative manner (i.e. group I of Shewan *et al.* 1960) is not clear.

Table 1. *A comparison of the effects of strains of* Pseudomonas *on various media*

Group and no. of strains		Production of opalescence* (a) and staining† (b) on				
		Egg yolk agar		Cream agar		Lecithin agar
		(a)	(b)	(a)	(b)	(a)
I	88	+	±	+	+	+
	6	—	—	—	—	—
II	4	—	—	—	—	—
	1	+	—	+	+	+
	1	—	—	+	+	—
III	20	—	—	—	—	—
	1	—	—	—	—	(+)
IV	8	—	—	—	—	—

* Reactions: +, opalescence or staining; —, no opalescence or staining; ±, opalescence with colony stained only in the centre; (+), faint opalescence.

† After flooding plate for 10 min with saturated copper sulphate solution.

The results of Rhodes (1959), however, indicate that she found a higher proportion of green fluorescent pseudomonads producing no action on egg yolk media (or other media containing fat) than the present authors. Paton (pers. comm.) has also found that green fluorescent pseudomonads found on plants are variable in their ability to break down margarine oil.

The general conclusion to be drawn from the results presented is that whereas the majority of strains of pseudomonads in group I produce both lipase and lecithinase, most of those in groups II, III and IV produce neither.

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A SELECTIVE MEDIUM FOR THE ISOLATION AND ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI FROM FOODS

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SUMMARY: A medium is described for the isolation and enumeration of coagulase positive staphylococci from food products. It was based on the observation that coagulase positive staphylococci produced a zone of clearing in egg yolk emulsion suspended in an agar medium. In conjunction with a modified coagulase test most coagulase positive staphylococci can be confirmed within 24 hr. The majority of coagulase negative staphylococci produced no clearing; when present it was frequently accompanied by a precipitation zone within the clearing area. Sodium azide was used as a selective agent.

Many types of media have been developed for the isolation and enumeration of coagulase positive staphylococci, but most of these have proved of limited value when dealing with isolations from foodstuffs. Much of this literature has been reviewed by Innes (1960).

Gillespie & Alder (1952) observed that 80% of coagulase positive staphylococci isolated from human sources produced an opacity in egg yolk broth whereas coagulase negative types failed to give this reaction. Carter (1960) utilized this reaction to produce a solid medium based on Chapman's 110 medium (Chapman, 1946) with the addition of brain heart infusion broth and egg yolk. Innes (1960) also utilized the egg yolk reaction in the preparation of a solid selective and differential medium for coagulase positive staphylococci.

This paper describes a selective plating medium incorporating egg yolk and sodium azide which has been successfully used in this laboratory for the isolation and enumeration of coagulase positive staphylococci.

Methods

Preparation of egg yolk-azide agar (EYAA). Concentrated egg yolk emulsion (Oxoid) was added to melted azide blood agar base (Difco) at 50° to give a final concentration of 5% (v/v). The resulting opaque medium had a pH of 6·8–7·0: at higher pH values the medium showed a progressive clearing and its diagnostic value was lost.

Treatment of material. Dried EYAA plates were surface inoculated by spreading 0·1 ml of dilutions of the food material under test and then incubated at 37° for 18–24 hr. Suspect colonies were tested for coagulase production.

Detection of coagulase. A modification of Fisk's tube coagulase method (Mackie & McCartney, 1953) was employed. Approximately 0·1 ml aliquots of nutrient broth (Oxoid no. 2) were pipetted into sterile 10×75 mm rimmed tubes. Suspensions of each of the suspect colonies were made in the broths, which were then incubated at 37° for 1 hr, when 0·3 ml of a 10^{-1} dilution of rabbit plasma (Burroughs Wellcome) in physiological saline was added to each of the tubes; these were then reincubated at 37° for a further 3 hr, during which period coagulase positive staphylococci clotted the plasma.

RESULTS AND DISCUSSION

The reactions of staphylococci on EYAA. Altogether, 235 staphylococcal isolates from foodstuffs gave clearing reactions in the EYAA medium. Colonies of all these isolates were subsequently tested for coagulase activity by the method described. The results, shown in Table 1, indicate that 77% of these isolates were coagulase positive staphylococci.

Table 1. *The coagulase reactions of isolates causing clearing of EYAA plates*

Source of isolates	No. clearing EYAA	No. with coagulase reaction	
		+	−
Comminuted meat products	161	127	34
Fish products	74	54	20

The colonies of coagulase positive staphylococci were surrounded by well defined clear zones whereas the majority of the coagulase negative isolates were recognized by the formation of an inner zone of precipitation immediately around the colonies (Plate 1). In the course of this work a similar number of non-egg yolk reacting colonies was examined for coagulase and none was found to be positive. Very occasionally colonies of coagulase positive staphylococci were seen which cleared the medium in 24–48 hr. Colonies of streptococci were frequently observed and were distinguished by their minute size and inability to clear the medium.

There was no evidence that sodium azide at the concentration used in the agar base inhibited the growth of coagulase positive staphylococci.

Quantitative comparisons of EYAA with a standard enrichment technique. The numbers of coagulase positive staphylococci present in 11 samples of artificially infected meat material were estimated using EYAA and a standard salt meat broth enrichment technique (Maitland & Martyn, 1948). The results, given in Table 2, show that there was good agreement between the counts obtained by these two methods. During the investigation all 50 colonies showing typical clearing zones were tested and found to be coagulase positive and 50 representative colonies showing clear zones with a ring of precipitation were confirmed as coagulase negative.

Table 2. *A comparison of results for meat samples tested by the salt enrichment method and counted on EYAA*

Sample no.	Coagulase + staphylococci detected by	
	Salt enrichment method (organisms present in, g)	EYAA method (count/0·001g)
1	0·001	1
2	0·0001	28
3	0·001	15
4	0·001	3
5	0·0001	24
6	0·0001	27
7	0·0001	52
8	0·0001	30
9	0·001	1
10	0·001	3
11	0·001	2

General remarks. It should be noted that the clearing produced by coagulase positive staphylococci in the EYAA medium is the reverse of the reactions reported by Gillespie & Alder (1952), Carter (1960) and Innes (1960) with their media. Carter's medium, which is dependent upon a higher salt concentration for selectivity, is clear and coagulase positive staphylococci produce opaque zones around the colonies, the coagulase negative strains giving no reaction. Innes (1960) reduced the opacity of his medium by incorporating 1% of sodium chloride and, in addition, the final pH was 8·0–8·2. On this medium coagulase positive staphylococci produced opaque or clear zones at 24 hr, the clear zones later becoming opaque.

The precise mechanisms of the reactions in EYAA are obscure and biochemical studies not undertaken in this work are needed.

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EXPLANATION OF PLATE

Plate 1. Appearance of colonies on EYAA (normal size).

A Coagulase positive colony, showing well defined zone of clearing.

B Coagulase negative colony, showing inner ring of precipitation.

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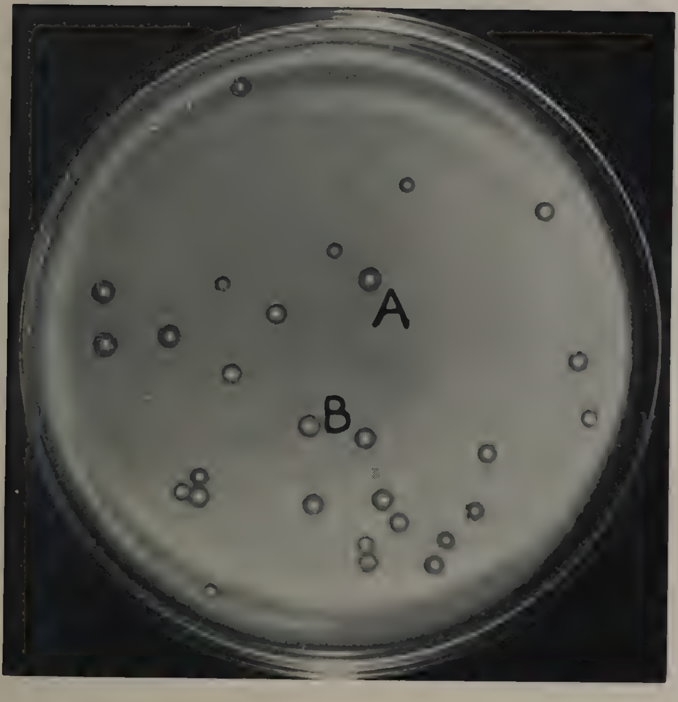

J. HOPTON — A SELECTIVE MEDIUM FOR THE ISOLATION AND ENUMERATION OF COAGULASE POSITIVE STAPHYLOCOCCI FROM FOODS. PLATE 1

FEISAL, E. V. & BENNETT, E. O. (1961). *J. appl. Bact.* **24** (2), 125–130.

THE EFFECT OF WATER HARDNESS ON THE GROWTH OF *PSEUDOMONAS AERUGINOSA* IN METAL CUTTING FLUIDS

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SUMMARY: An investigation of the effect of the salts found in hard water on the growth of *Pseudomonas aeruginosa* in metal cutting fluids is reported. It was found that hard water and the salts found in hard water stimulate the growth of this organism in the two products employed in the study.

To DATE, only a few reports concerning the effects of inorganic salts on hydrocarbon oxidation by micro-organisms have appeared in the literature. Strawinski & Stone (1954) observed that when calcium chloride and copper sulphate were omitted from the basal medium, there was a reduction in the oxidation of naphthalene by *Pseudomonas aeruginosa*. These workers also noted that 0·1–0·5% of calcium carbonate exerted no appreciable effect upon oxidation of the compound and that the addition of disodium hydrogen phosphate in concentrations above 0·25% exerted an inhibitory effect. Ladd (1956) found that a 0·0008 M concentration of potassium ions stimulated the oxidation of *n*-decane, *n*-hexadecane, and *n*-tetradecane by a *Corynebacterium* sp. The same concentration of ammonium ions resulted in a greater activity than was noted with potassium ions when *n*-hexadecane was used as a carbon source.

These reports indicate that certain inorganic salts may have a significant effect on the oxidation of hydrocarbons by micro-organisms. If the importance of inorganic salts were clearly understood, this information could be of value in the field of petrochemical synthesis using micro-organisms. In addition, the information could be used to control the microbial deterioration of petroleum products.

The objective of this investigation was to determine the effects of the salts commonly found in hard water on the growth of *Ps. aeruginosa* in two different types of cutting fluid.

EXPERIMENTAL PROCEDURE

A culture of *Ps. aeruginosa* isolated from a cutting fluid was used throughout this investigation. The culture was maintained on trypticase-soy agar under refrigeration and examined for purity at regular intervals. The stock culture was used to inoculate sterile Kolle flasks containing a medium composed of: Bacto peptone, 20 g; glucose, 10 g; sodium sulphate, 1·5 g; magnesium sulphate, 1·5 g; agar, 15 g; in 1 l. of distilled water. The Kolle flasks were incubated at 37° for 24 hr and the cells were then harvested and washed in distilled water using aseptic procedures. The cell suspension was standardized to an optical density of 1·0 at 400–460 mμ, using a Klett-Summerson photoelectric colorimeter.

The products, a chemical coolant designated as cutting fluid A and an emulsion oil designated as cutting fluid B, were prepared in double distilled water, dispensed into 250 ml Erlenmeyer flasks, and autoclaved for 15 min at 121°. After the flasks had cooled, sterile salts solutions were added to each flask in the required concentration. All salt solutions were made in double distilled water using the purest obtainable chemicals and were sterilized by filtration through a fritted glass filter (UF). The flasks were made up so that they would contain a final cutting fluid concentration of 4%, the desired concentration of salts, and 1 ml of the standardized cell suspension in a total volume of 100 ml. The flasks were incubated at 37° on a rotary shaker at 180 rev/min. Standard plate counts were made in duplicate at appropriate intervals during the test period and the plates were incubated for 24 hr at 37° before they were counted.

The synthetic hard water was prepared by adding 1·5 g of $CaCO_3$ to 100 ml of double distilled water and adding dilute HCl until the solution cleared completely. To this solution 1·5 g of $MgSO_4$ were added and the total volume was diluted to 4 l. with double distilled water. After dilution, 100 ml of double distilled water containing 1·3 g of NaCl were added. This mixture resulted in water with a total hardness of 700 p/m.

The synthetic soft water was prepared by diluting 400 ml of the synthetic hard water to 4 l. and adding 100 ml of double distilled water containing 5·6 g of NaCl. This water had a total hardness of 72 p/m.

The experiments and plate counts were done in duplicate and appropriate controls were included in each experiment. The experiments were repeated several times and representative results are given in the Tables.

RESULTS

Tables 1–4 show that $CaCl_2$, $MgCl_2$, $CaSO_4$, and $MgSO_4$ had a significant effect upon the growth of pseudomonads in both cutting fluids. Magnesium chloride appeared to have the greatest stimulatory effect whereas calcium chloride had the least effect. The optimal concentration of all four salts appeared to be approximately 1,000 p/m.

Table 1. *The effect of calcium chloride on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $CaCl_2$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·10	0·11	1·50	2·42	2·65	2·90	2·46	3·11	3·62	4·86
250	0·18	0·15	1·15	3·10	2·90	7·30	3·73	9·80	5·45	10·55
500	0·14	0·09	2·33	2·68	3·31	5·30	3·56	8·50	5·69	9·78
1,000	0·10	0·18	2·45	2·80	3·49	4·67	4·86	9·70	6·58	10·20
1,500	0·11	0·12	2·30	1·56	3·55	3·10	4·99	6·30	4·89	9·34
2,000	0·08	0·07	2·48	1·85	3·63	2·58	3·84	7·54	4·78	6·80
4,000	0·17	0·15	1·67	2·20	2·99	4·22	3·98	5·95	5·98	8·61

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 2. *The effect of magnesium chloride on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $MgCl_2$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·12	0·07	1·18	1·72	2·32	3·40	2·43	4·18	2·64	6·80
250	0·09	0·12	2·22	2·21	3·56	3·55	4·94	8·00	5·15	9·70
500	0·17	0·19	2·21	2·07	2·81	4·45	4·18	7·20	5·35	11·50
1,000	0·14	0·21	1·76	2·32	3·57	5·34	4·52	8·95	6·76	13·46
1,500	0·11	0·12	2·23	3·66	3·94	7·10	5·01	10·90	5·46	14·86
2,000	0·06	0·09	2·39	4·05	3·66	5·60	5·99	7·89	4·97	8·62
4,000	0·18	0·10	2·56	2·44	3·15	4·76	4·79	6·99	5·48	9·77

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 3. *The effect of calcium sulphate on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $CaSO_4$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·09	0·19	1·20	3·86	1·80	4·98	2·80	6·00	3·51	6·80
250	0·18	0·10	2·64	4·10	2·50	5·31	3·40	6·58	3·80	8·70
500	0·11	0·14	2·83	5·60	3·21	5·78	4·30	7·40	4·90	8·96
1,000	0·15	0·18	1·64	7·86	2·72	8·16	5·42	10·80	5·80	13·40
1,500	0·10	0·14	1·40	5·40	2·20	9·60	4·00	11·00	6·11	15·70
2,000	0·16	0·08	0·94	5·66	1·60	10·00	2·62	8·61	3·20	10·72
4,000	0·09	0·10	0·50	3·11	1·10	5·41	2·40	6·80	3·00	7·46

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 4. *The effect of magnesium sulphate on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $MgSO_4$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·09	0·19	1.20	3·86	1·80	4·98	2·81	6·00	3·51	6·80
250	0·10	0·10	2·56	3·77	2·80	4·70	3·69	6·20	3·90	8·00
500	0·14	0·09	2·12	4·80	3·46	5·62	5·20	7·31	5·89	10·00
1,000	0·08	0·14	2·44	6·70	4·80	7·86	4·91	13·60	5·70	15·60
1,500	0·16	0·08	2·10	6·68	3·40	9·89	4·28	10·80	6·40	12·78
2,000	0·11	0·13	2·66	5·40	2·31	6·70	3·80	7·52	5·90	10·50
4,000	0·17	0·11	1·90	3·16	1·16	4·70	2·56	6·10	2·20	8·50

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Tables 5 and 6 show that calcium and magnesium carbonates produced a quite different effect from the chlorides and sulphates. The latter salts increased microbial growth in concentrations ranging from 250–1,500 p/m, but in concentrations from 2,000–4,000 p/m they inhibited growth.

Table 5. *The effect of calcium carbonate on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $CaCO_3$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·18	0·19	2·10	3·86	2·87	4·98	3·69	6·00	5·14	6·80
250	0·11	0·10	2·31	2·84	3·56	3·98	3·39	5·40	4·20	6·94
500	0·09	0·07	2·29	3·10	3·01	4·10	4·10	6·73	7·00	7·00
1,000	0·13	0·13	2·93	3·89	3·25	6·89	5·76	7·50	8·18	8·19
1,500	0·07	0·10	2·55	3·26	3·99	7·40	5·44	6·46	6·79	6·70
2,000	0·12	0·09	1·89	2·32	1·54	3·48	2·10	3·20	3·55	5·32
4,000	0·18	0·17	1·30	1·89	1·66	2·80	2·62	2·40	2·10	3·11

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 6. *The effect of magnesium carbonate on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $MgCO_3$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days)									
	0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·18	0·19	2·10	3·86	2·87	4·98	3·69	6·00	5·14	6·80
250	0·08	0·11	2·27	2·91	3·10	5·30	3·98	6·98	5·70	7·20
500	0·15	0·09	3·32	3·34	3·80	5·92	4·23	8·80	6·99	8·00
1,000	0·11	0·15	2·61	3·48	4·89	5·60	5·15	6·26	8·10	7·40
1,500	0·18	0·19	2·85	3·68	3·20	5·63	5·40	5·26	6·00	6·84
2,000	0·09	0·10	2·19	2·80	1·50	3·20	2·78	4·90	3·50	3·57
4,000	0·12	0·17	1·86	1·70	1·10	2·30	1·90	3·29	2·85	4·83

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 7 shows the effect of $NaHCO_3$ on the growth of pseudomonads in the cutting fluids. This compound appeared to inhibit growth slightly in the products.

The effects of distilled water, soft water, and hard water on growth may be seen in Table 8. It may be noted that as the salt content of the water increased the microbial growth also increased. It is interesting to note that growth occurred even when distilled water was used. This observation indicates that sufficient concentrations of essential ions necessary for growth were present in the concentrated products.

The results clearly show that the salts had a greater effect upon the growth of *Ps. aeruginosa* in the emulsion oil than in the chemical cutting fluid.

Table 7. *The effect of sodium bicarbonate on the growth of* Ps. aeruginosa *in two cutting fluids**

Concn of $NaHCO_3$ (p/m)	No. of organisms† (10^6/ml) after incubation for (days) 0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
0	0·11	0·09	1·91	2·74	2·44	3·89	3·87	6·95	5·89	8·42
250	0·17	0·15	1·74	2·89	2·65	3·60	3·00	7·10	5·44	7·98
500	0·08	0·11	1·20	2·10	2·00	4·11	2·98	6·44	4·66	8·00
1,000	0·15	0·19	1·81	2·34	3·11	3·10	3·43	6·88	5·76	7·65
1,500	0·12	0·14	1·11	3·10	1·89	4·11	3·20	6·55	4·62	7·14
2,000	0·07	0·07	1·89	2·23	2·22	3·65	3·65	6·92	6·00	6·72
4,000	0·16	0·18	1·40	2·63	2·13	3·47	3·31	6·42	4·90	7·55

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

Table 8. *The effect of different waters on the growth of* Ps. aeruginosa *in two cutting fluids**

Type of water	No. of organisms† (10^6/ml) after incubation for (days) 0		1		2		3		4	
	A	B	A	B	A	B	A	B	A	B
Distilled	0·18	0·16	1·20	2·89	1·56	4·56	2·20	8·64	4·89	9·19
Soft‡	0·11	0·18	3·46	14·73	5·50	10·22	6·14	13·21	6·78	15·90
Hard§	0·15	0·12	3·11	10·50	8·17	16·32	9·81	21·00	9·17	33·45

* Cutting fluids designated A and B, respectively (see text).

† Average values of two determinations made on each duplicate flask by standard plate count techniques.

‡ Total hardness equivalent to 72 p/m of $CaCO_3$.

§ Total hardness equivalent to 692 p/m of $CaCO_3$.

DISCUSSION

The results of the present investigation show that the presence of certain inorganic salts can have a significant effect upon the growth of *Ps. aeruginosa* in cutting fluids. Each of the salts, with the exception of $NaHCO_3$, stimulated growth of the organism. However, the mixture of salts produced greater stimulation than any of the individual components.

It may be noted that provided a salt is present its concentration appears to be unimportant. This observation may suggest that absolute concentration is not as critical in stimulating growth as is the relative number and types of ions present in the environment, and supports the observations of other workers. Ladd (1956) observed that no further stimulation of hydrocarbon oxidation by a *Corynebacterium* sp. occurred when the concentration of potassium was increased by as much as tenfold. McLeod & Snell (1948) stated that the ratio of dissociated ions present in a medium, rather than an exact concentration of salts, influenced metabolism.

It can be observed that the chemical coolant (product A) did not support growth as well as the emulsion oil and in many instances the inorganic salts did not have as great an effect in the chemical coolant. Sluhan (1960) reported previously that the chemical coolants were more resistant to microbial growth and were not affected as much by hard water.

Inorganic salts may have an additional undesirable effect upon the deterioration of petroleum products, as it has been reported that the salts found in hard water can reduce the effectiveness of several types of antimicrobial agent. The inhibitory activity of amines (MacLeod & Onofrey, 1954), cationic detergents (Armstrong. 1958), quinoline (Gale, 1949), quaternary ammonium compounds (Cousins & Clegg, 1956) and phenols (Kravitz & Stedman, 1957), to name a few, are reduced in the presence of the salts found in hard water. Thus, it may be seen that hard water can have a twofold effect: first, it can reduce the effectiveness of the antimicrobial agent; and second, it can stimulate microbial growth.

It is not surprising to note that the most critical problems pertaining to the deterioration of metal cutting fluids are encountered in areas where hard water is used to make the coolant. The results of this study indicate that a simple method of combating spoilage and deterioration of cutting fluids by micro-organisms would be to use water from which these deleterious ions have been removed.

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A MULTIPLE INOCULATION DEVICE FOR USE WITH FLUIDS

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SUMMARY: A multiple inoculation device comprising a stainless steel inoculator and Perspex containers for inocula is described. It has been used mainly in the screening of different strains of bacteria for lysogeny, but other applications are listed. Comparison is made with other similar devices.

THE MULTIPLE inoculation apparatus to be described was designed for the purpose of screening a number of strains of *Pasteurella pestis* for lysogeny (Smith, 1961*a*). It can be regarded as an adaptation of the replica plating method (Lederberg & Lederberg, 1952; Braendle & Szybalski, 1957; Eversole, 1956; Roberts, 1959) to fluids, for speeding up a process otherwise performed with a loop and for greatly reducing chances of contamination.

Description

The device consists of two parts, the multiple inoculator and a number of containers for liquid inocula (Plate 1a, b). The inoculator is made of a circular stainless steel plate (FDP grade) 0·3 cm thick and 8·5 cm in diameter fitted on the upper side with a centrally placed handle of stainless steel rod 7·5×0·6 cm. Into each of 16 symmetrically arranged tapped holes in this plate is fitted a 1·5 cm length of 2BA stainless steel bolt with the thread removed from the lower half and a screwdriver slot cut in the threaded end. The distance the turned ends of the bolts protrude is thus adjustable; normally it is about 0·5 cm from the lower end of the plate. These bolts constitute the inoculation stubs. Each is locked in place with a brass nut which bears on the upper surface of the plate. The containers for inocula consist of 8 cm diameter discs of Perspex sheet (Imperial Chemical Industries, Welwyn Garden City, Herts.) of 1·5 cm thickness, drilled to give 16 rounded wells 1·4 cm in diameter and 0·7 cm deep at their deepest point, and concentrically aligned with the multiple inoculator stubs. Each disc, with its wells uppermost, is covered by the lid of a 9 cm diam. Petri dish.

The inoculation stubs are sterilized by dipping the inoculator in 20 ml of 70% alcohol in a Petri dish and then flaming its underside. The containers are sterilized by immersion in 1–2% Cetavlon (Imperial Chemical Industries, Wilmslow, Cheshire) for at least 12 hr. They are then washed in hot water, rinsed in distilled water, dried, fitted with Petri dish lids and finally heated in an oven at 60° for 1 hr.

About 0·3 ml of suspensions to be tested is pipetted into each of the wells. The 16 separate drops (each of about 0·01 ml volume) attached to the stubs of the sterilized inoculator are transferred from the wells to appropriate agar plates. Each drop covers an area 0·5–0·7 cm in diameter. For each of a series of plates already seeded with bacteria (or phage) the inoculator is resterilized and the process repeated. Thirty plates can be inoculated before the wells need refilling.

Application

The efficiency of the device for the detection of lysogeny was tested using two established lysogenic systems. First, broth cultures of *Escherichia coli* K12 lysogenic for phage λ (Lederberg & Lederberg, 1953) and the indicator strain C600 were inoculated from different container wells to 'lawns' of *c*. 2×10^8 organisms of strain C600 on the surface of agar plates. After 17 hr at 37°, lysogenic inocula were clearly distinguishable from nonlysogenic. Second, serial dilutions of a lysogenic culture of *Salmonella typhi-murium* LT-2 (Zinder & Lederberg, 1952) were plated on nonlysogenic LT-2. The presence of lysogenic organisms down to 10^3–10^4/ml could be detected after incubation for 17 hr.

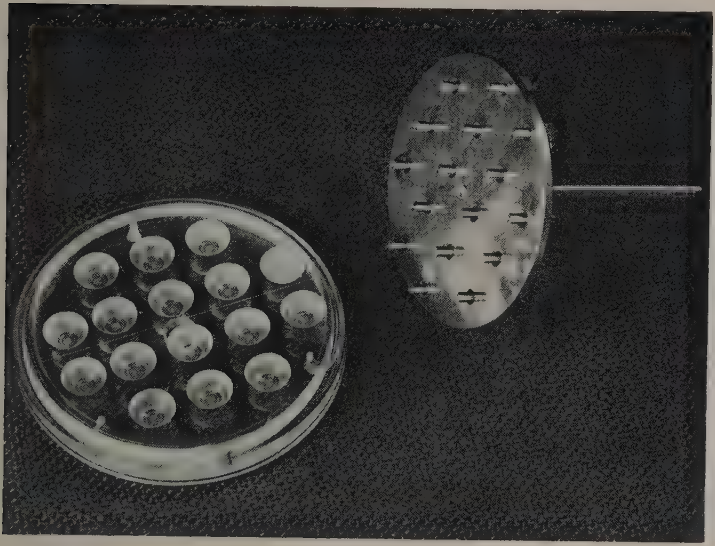
In attempts to detect lysogeny among strains of *P. pestis* (Smith, 1961*a*), drops of broth cultures of 54 different strains were placed on 'lawns' of all of these strains as potential indicators. The experiments were repeated using similar cultures of all the strains which had been irradiated with ultraviolet light and the number of potential indicators increased to 70. Over 6,500 combinations were achieved in this way.

The device has also been used successfully to determine the host range and efficiency of plating of a number of phages lytic for *P. pestis* (Smith, 1961*a*), the growth responses of methionineless mutants of *S. typhi-murium* (Smith, 1961*b*), the transducibility of different auxotrophs of the same organism with different phage preparations, the mating types of derivatives of *E. coli* K12 and the sensitivity of certain strains of bacteria to bacteriocins.

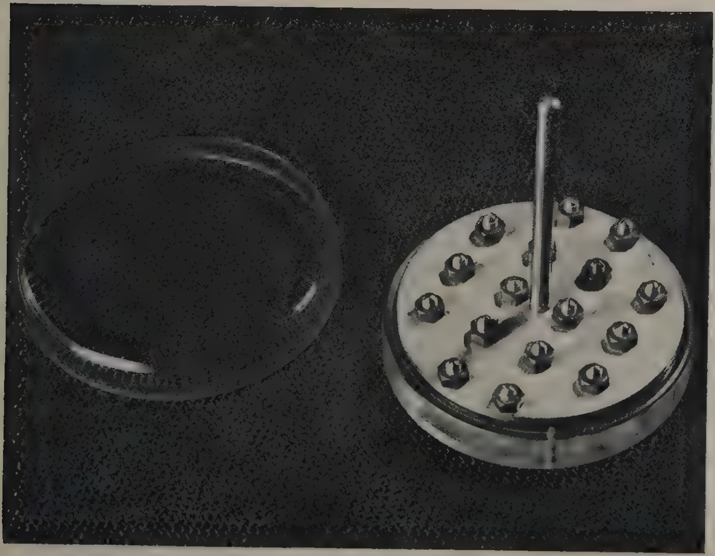
DISCUSSION

Beech, Carr & Codner (1955) described a multipoint inoculator applicable to fluid cultures of yeasts or bacteria which Welsch (1959) used in a slightly modified form for the detection of lysogeny in actinomycetes. It seems less simple to construct and manipulate than the device described above and quick sterilization of the inoculator (between inoculations) would be difficult if not impossible. The zinc gauze and bolt instrument used by Holliday (1956) for multiple inoculation from colonies of *Ustilago maydis* on a master plate could probably be used for fluids, and therefore compared with the stainless steel inoculator, except that it would be less durable. Designed specifically for the phage typing of *Staphylococcus aureus*, the apparatus described by Tarr (1958) also appears less durable and is more complicated, e.g. it involves the use of racks of tubes instead of sterilizable Perspex discs. Tarr's movable inoculating loops probably ensure no indentation of the surface of the agar. However, the marks made by the stainless steel inoculating stubs do accurately locate the sites of inoculation.

The author is indebted to Mr. G. Clements for making the multiple inoculation device.



(a)



(b)

D. A. SMITH — A MULTIPLE INOCULATION DEVICE FOR USE WITH FLUIDS. PLATE 1

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EXPLANATION OF PLATE

PLATE 1. A multiple inoculation device.

(a) The inoculator and a container for liquid inocula shown separately (× $\frac{1}{2}$).

(b) The inoculator in position with its stubs immersed in the wells of a container (× $\frac{1}{2}$).

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AN EPIDEMIC AMONG INFANTS CAUSED BY *SALMONELLA MUENCHEN*

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SUMMARY: An account is given of a *Salmonella muenchen* epidemic among infants which was disseminated through an industrially produced and marketed cereal preparation for infant consumption. The epidemic continued for 6 months and was distributed over the whole country. The total number of cases was 110, of which the 62 primary cases were infants under the age of 2 years who were being bottle fed. The duration of excretion of bacteria was studied for both primary and secondary cases grouped according to age. The bacteriological examinations carried out support the view that a small number of *Salm. muenchen* cells is sufficient to give rise to illness in infants. With the low degree of infection of the cereal powder it proved difficult with the usual technique of cultivation to show the presence of salmonellae and this technique was therefore modified.

Salmonella epidemics among infants are very rare in Sweden, and the one to be described in this paper is thus of particular epidemiological interest. The food on which an infant subsists is different from that consumed by older children and adults; the channels of infection among infants thus occupy a special position from the epidemiological point of view.

During the years 1935–54 only 64 cases of *Salmonella muenchen* infection were diagnosed in this country, 63 of these occurring in 1953 in connection with the importation of South American meat (Lundbeck, Plazikowski & Silverstolpe, 1953). *Salm. muenchen* is thus rare in Sweden and had not previously given rise to epidemics.

Characteristics of the epidemic

Course of the epidemic. The first case in this epidemic (Silverstolpe & Plazikowski, 1955), a boy aged 7 months, fell ill in Gothenburg in the middle of April, 1955. During the subsequent four months several cases occurred, which were also among infants and were scattered over the greater part of the country. The epidemic lasted a month and culminated during the first part of May, gradually subsiding at the end of August and the beginning of September of the same year. The cases occurred from Kronoberg County in the south up to Norrbotten County in the north. During the six months the epidemic lasted, 110 cases were diagnosed, 62 being primary cases distributed over practically the entire country (Table 1). Of these, 28 cases occurred in Stockholm and 30 in Gothenburg (Fig. 1).

Table 1. *The distribution of* Salmonella muenchen *infection in* 1955 *according to age and sex*

Age (years)	Sex		Total
	Male	Female	
<2	36	26	62
2–14	4	9	13
>14	15	20	35
Total	55	55	110

Clinical picture. All the primary cases (62) were infants under two years of age, and all except one (for which data were not available) had symptoms in the form of diarrhoea and vomiting. Of 61 sick children (under 2 years of age), 21 were severely affected and required the parenteral administration of fluids. Of the siblings of the primary cases (13 cases aged 2–14 years) who were secondarily infected, only about 50% had clinical symptoms; these were rather mild and of relatively short duration. The others in this age group were symptom-free excreters of the organisms. The

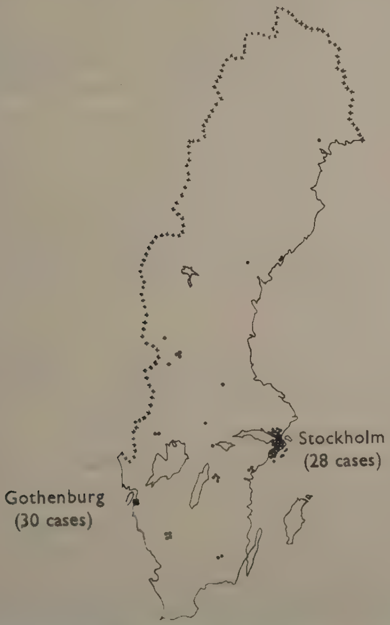

Fig. 1. The distribution of cases of *Salmonella muenchen* infection in Sweden in 1955. Each spot indicates a single case.

secondary cases among older siblings (over 14 years of age) and other household members (35 cases) had, in 40% of cases, very mild clinical symptoms. The other cases in this group were also symptom-free excreters. The clinically ill cases and healthy excreters (a total of 58 cases) in the cities of Stockholm and Gothenburg are given in Table 2.

Table 2. *The incidence of clinically ill* (*a*) *and healthy* (*b*) *excreters among* Salmonella muenchen *cases* in* 1955

Age (years)		Sex				Total
		Male		Female		
		(a)	(b)	(a)	(b)	
<2		1	14	0	10	25
2–14		1	3	4	4	12
>14		6	2	6	7	21
	Total	8	19	10	21	58

* Only the Stockholm and Gothenburg cases.

One fatal case was reported which might possibly have been due to *Salm. muenchen*. This was a boy 6 months old who fell ill in the same way as the others, namely as the result of the consumption of an infants' cereal. He suffered seriously from severe diarrhoea, vomiting and high fever. The parents refused to allow the child to be taken to hospital and the child died at home after a brief illness. A 4-year old brother fell ill with the same symptoms only a few days later, but was not seriously affected. No sample of faeces was taken in either case, and a Widal test done later on the 4-year old boy was negative.

Excretion of bacteria. The excretion of salmonellae by the infected infants was investigated, and the results in the various age groups compared (Fig. 2). After the first positive faecal sample had been diagnosed, further samples were taken about every third day, until five negative samples had been obtained; the patients were then discharged. The period for the excretion of salmonellae proved to be longest for the primary cases, with an average of 5½ weeks and a maximum of 15 weeks. For secondary cases in the age group 2–14 years it was on an average 4 weeks with a maximum of 10 weeks, and for the age group over 14 years an average of 1 week and a maximum of 7 weeks. The primary cases were excreters for more than twice as long as the secondary cases in the group over 14 years of age, in which the majority were symptom-free temporary carriers. These findings are comparable with those obtained from the salmonella infections in Östergötland County in Sweden in the year 1953 (Silverstolpe & Wranne, 1955), where it was found that infected individuals with clinical symptoms excreted bacteria for a considerably longer time than did symptom-free excreters.

As regards the period of incubation, no reliable information concerning the primary cases was obtainable. This was due to the fact that the cereal preparation in question had been used for a considerable time. For the secondary cases the shortest incubation period was 2 days.

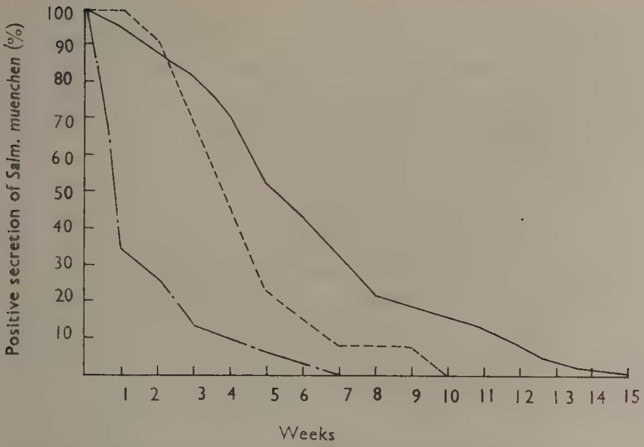

Fig. 2. The duration of excretion of *Salmonella muenchen* by patients grouped according to age.
Continuous line, data for 61 patients less than 2 years old; chain dots, data for 13 patients 2–14 years old; dashes, data for 35 patients more than 14 years old.

Distribution according to age and sex. All the 62 primary cases were bottle fed and comprised 56% of the total cases. Of these, 36 were boys (56·5% of the primary cases) and 26 girls (43·5%) (Table 1). Since the exposed population was not known, it is not possible to state with certainty that there was any increased incidence among boys. The secondary cases showed, in the higher age group, an increased morbidity in women, owing to the fact that they come into such close contact with infants.

Incidence of secondary infection. Eighteen families with one primary case each were examined. The number of contacts and the number of persons contracting the disease are recorded in Table 3. The incidence of secondary attack was 44%, a high figure which reflects the massive focus of infection constituted by a salmonella-infected child with diarrhoea.

Table 3. *The number of secondary cases occurring in* 18 *families each with one primary case of* Salmonella muenchen *infection*

No. of primary cases	Total members in families	No. contracting the disease	Secondary attack (%)
18	66	29	44

Source of infection. The epidemiological investigation showed a clear connection between a cereal powder sold on the market and the primary cases, all of whom had faecal samples positive for *Salm. muenchen*. All these cases had, moreover, eaten the cereal preparation in question. It was also possible to show the presence of *Salm. muenchen* in packets of this cereal that had not previously been opened.

Preparation of the cereal. The cereal powder in question is intended for infant consumption. It is supposed to be mixed with water (1 part of powder to 2 parts of water) at a temperature suitable for drinking immediately as a gruel. Thus according to the instructions the cereal is not to be boiled. It is probable that a number of mothers prepare all the gruel they require for a day in one operation and this may permit bacterial growth to occur in prepared gruel.

Bacteriological Examinations

Examination of faecal samples. The technique applied for the examination of faecal samples during the epidemic was as follows: first, enrichment was carried out in the medium of Kauffmann (1954), incubated for 24 hr at 37°. This was transferred to deoxycholate-citrate agar plates and incubated for 48 hr at 37°. Finally, typical colonies were subcultured on to Endo agar plates, then typed by fermentation and serological methods.

Examination of cereal. Despite the fact that all the evidence supported the assumption that the cereal powder was infected with salmonellae, it was not possible to show the presence of these organisms by the routine methods of cultivation. Experiments were therefore done to find a suitable method for the cultivation of the salmonellae in the cereal powder, as it was evidently a matter of a low degree of infection. Parallel experiments were made with 200 ml flasks containing each of the following: bile substrate, Kauffmann broth, selenite broth, physiological saline, and distilled water. To each 200 ml flask 100 g of cereal powder were added and the mixture was inoculated with a small number (5–20) of *Salm. muenchen* cells. They were then cultivated in an incubator at 37° for 24 hr. It was found that only the flasks with distilled water and cereal powder gave growth of bacteria. That no growth occurred in the other flasks may possibly be ascribed to the increased salt content resulting from the addition of the cereal powder. The flasks with distilled water and cereal powder thus provided the best medium for growth.

The following technique was accordingly adopted for the cultivation of salmonellae present in cereal powder. A mixture of 300 ml of distilled water and 100 g of cereal powder was left for 24 hr in an incubator at 37°. Then 0·5–1 ml was transferred to deoxycholate-citrate agar, after which the technique described above for the faecal samples was applied. Two examinations were carried out, one qualitative (to ascertain which part of the date-stamped production of cereal was infected with salmonellae) and the other quantitative (to determine the degree of infection with salmonellae, coli-aerogenes bacteria and enterococci).

The rate of growth of *Salm. muenchen* was also studied. One litre of prepared cereal (corresponding approximately to one day's requirement for one infant) was inoculated with a few (1–5) *Salm. muenchen* cells. The cereal was allowed to stand at room temperature (20°) and samples were taken, after careful mixing, after 3, 5, 7, 9 and 24 hr.

Investigation of the channels of infection. The investigation was directed towards finding out how the cereal became infected, whether through handling of the powder or through infected raw materials.

RESULTS

Characteristics of the strain of Salm. muenchen *causing the epidemic.* The antigenic formula of the epidemic strain was 6, 8: d, 1, 2. Arabinose, dulcitol, inositol, maltose and mannitol were rapidly fermented with the production of gas; rhamnose, xylose, and sorbitol were fermented without gas formation; lactose and sucrose were not fermented. Positive reactions were obtained in the H_2S and methyl red tests and in Stern's glycerol-fuchsin medium (Kauffmann, 1954). No growth was obtained in D-tartrate medium.

Examination of the cereal product. Of 137 packets of cereal examined from the whole period of production, 34 proved to be positive for *Salm. muenchen* (24·8%). All of these were manufactured during a given period, namely between March 17 and April 14, 1955. The examination was carried out two months after the date of production.

Quantitative examinations of the cereal powder for coli-aerogenes bacteria and enterococci were carried out on 28 packets from the infected consignment and on 23 packets of the noninfected product. The packets had been stored for about 5 months. The results are given in Table 4. It was found that the numbers of these organisms in the cereal powder were considerably greater during the period when the salmonella infection occurred than during other manufacturing periods. It is remarkable that no coli-aerogenes bacteria could be shown in 4 of the packets containing *Salm. muenchen.*

Table 4. *The occurrence of salmonellae, coli-aerogenes bacteria (a) and enterococci (b) in baby cereal*

Condition of consignment	No. of packets examined	No. from which *Salm. muenchen* was isolated	No. of bacteria/10 g in the ranges							
			0		1–10		11–50		>50	
			(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Infected	28	4*	7	5	14	13	7	8	0	2
Noninfected	23	0	18	10	4	13	1	0	0	0

* None of these contained coli-aerogenes bacteria.

On renewed examination of 100 g of cereal powder from 62 new packets 5 months after the date of manufacture, only 4 packets were positive (6·5%). From the 4 positive packets a further 100 g were immediately taken for a further examination for salmonellae. These proved to be negative for salmonellae. These examinations support the assumptions that during the 3 months elapsing between the two examinations a large number of salmonellae had died, and that the degree of infection in the cereal powder was low.

Only one of 18 mothers questioned stated that she prepared all the cereal needed for the day at one time. The others maintained that they had prepared the cereal immediately before it was required. The result of this inquiry thus indicated that, in the majority of the primary cases, the infecting dose must have been low.

The experiment done on the rate of growth of *Salm. muenchen* in cereal showed that after 9 hr a normal meal of the cereal (200 g) still contained fewer than 100 salmonellae (Table 5).

Channels of infection. Faecal samples were taken from all employees at the factory, and also from the staff of the firms supplying the raw materials. All the faecal samples yielded negative results for salmonellae. The raw materials used in the cereal powder were routinely examined at the factory for coli-aerogenes bacteria and total organisms. The same procedure was also applied with all the other products manufactured. No striking results were obtained, but routine examinations for salmonellae had not been carried out. The water used at the factory was also examined routinely and proved to be normal, with no increased coli-aerogenes content during the period relating to the epidemic. The general hygienic conditions in the factory did not warrant any undue criticism.

Table 5. *The rate of growth* of* Salm. muenchen *in prepared gruel at about* 20°

Time (hr)	No./100 g of cereal
3	1
5	2
7	8
9	40
24	1×10^6

* The results are means of 3 experiments.

The normal procedure for the manufacture of the cereal product was as follows. The raw materials, consisting of dried milk, flour, oatmeal, potato meal, malt diastase, sugar, salts and vitamins, were heat treated in the factory to a temperature sufficient to satisfy hygienic standards, with the exception of the vitamins, which were obtained in a sterile form from a drug factory. Mixing in certain proportions was then effected in a rotating mixer.

There did not seem to be any possibility of an infection being introduced into the cereal powder under these apparently satisfactory conditions, so it remained to be seen whether there had been any change in the mode of manufacture during the period March 17–April 14. This proved to have been the case. During this period oatmeal was used which had been treated in a different way from usual. After steam preparation of the oats they were ground in a mill, then mixed into the cereal powder without further heating. The steam preparation of the oatmeal took place under pressure and at a high temperature (over 100°), and was certainly satisfactory from the bacteriological point of view. There thus remained the mill as a source of infection. This had previously been used for grinding some malted barley meal which was subsequently rejected on account of a too high coli-aerogenes content, discovered by the laboratory attached to the cereal factory. However, no examination for salmonellae had been carried out at the factory. All this malt meal had been thrown away so that none was available for examination at the time of our investigation. Salmonellae were not found in the remaining small portion of oatmeal (a reference sample of about 10 g) nor in waste from the mill, though this can be explained in the light of the very small amount available for examination. The waste from the mill, however, contained 30,000 coli-aerogenes bacteria/g, and the oatmeal 200/g. The malt manufacturer had nothing left of the original malted barley, which was of Swedish origin. However, there was an African consignment that had just been ground, and in this it was possible to show the presence of *Salm. gatuni*, and also a rather high coli-aerogenes content of 20,000/g. Immediately before the malting of the Swedish barley subsequently used in the infected infant cereal another African consignment of barley had been malted in the same vat. It is probable that this barley was the source of the *Salm. muenchen*, especially as on the occasion of the investigation it was possible to show the presence of salmonellae in another consignment of African barley. Even if the chain of proof is not completely closed, it is nevertheless feasible to sketch the probable course of infection as follows. A consignment of African barley was the source of *Salm. muenchen*. During malting this infected a Swedish barley consignment, which in its turn infected the mill with which the oatmeal for the infants' cereal was ground. The infection was introduced into the cereal powder when the oatmeal was mixed with it without prior heating. This procedure lasted only during the period March 17–April 14 and was replaced by the old method as from May 15. The factory abandoned the steamed oatmeal because it resulted in a cereal of undesirable consistency. Instead, the factory reverted to the old technique of heating all the oatmeal to a satisfactory temperature before mixing it with the cereal powder. This agrees well with the bacteriological examination of the product which indicated *Salm. muenchen* infection and increased coli-aerogenes organisms in packets only during the manufacturing period mentioned above.

Discussion

The epidemic described here throws light on many questions of an epidemiological and hygienic nature. The outbreak illustrates the risks connected with a centralized industrial manufacture of foodstuffs. This applies especially to foodstuffs which according to the manufacturer's instructions are intended for infant consumption without the security afforded by sufficient heating. Manufacture of such foodstuffs is a very delicate matter, especially as the foods often constitute an excellent substrate for the growth of bacteria. Manufacture of this kind must therefore be subjected to particularly careful bacteriological and hygienic control (Vahlne & Kjellander, 1956). In such controls the total number of bacteria and the number of coli-aerogenes bacteria are generally used as criteria of the degree of contamination. But the number of coli-aerogenes bacteria does not always give a correct picture of the bacteriological standard of a food. The investigations of Thomas & Hobson (1954) among others have shown that ripening cereal seeds contain appreciable numbers of coli-aerogenes and other bacteria, including heat resistant organisms, without necessarily constituting a source of infection. On the other hand, it is known that eggs or egg powder infected with salmonellae may be free from coli-aerogenes bacteria. In the *Salm. muenchen* epidemic reported here it proved that the packets of cereal infected with salmonellae varied considerably in their content of coli-aerogenes bacteria, some packets containing none of these organisms. Examination for coli-aerogenes bacteria should thus always be supplemented with an examination to determine the presence of pathogenic bacteria, including salmonellae. Such examinations were not carried out as a matter of routine in the case of the cereal powder described above.

It is impossible to say, after the event, whether the epidemic referred to here might have been prevented if the ingredients of the cereal powder had been subjected to an examination for salmonellae. The degree of infection of the powder was obviously rather low, and it is possible that a random sample taken for control would not have revealed the infection. Only a careful hygienic control not only of the foodstuff factories, but also their suppliers of raw materials (in which case the degree of infection, if present, would be considerably higher and consequently easier to trace) has any prospect of making the preventive measures effective. The necessity for hygienic supervision has been frequently pointed out recently in connection with epidemics (e.g. Andersson & Stone, 1955; Lundbeck, Plazikowski & Silverstolpe, 1955; Mossel, 1956).

As regards adults, it is known that the infection dose for most *Salmonella* spp. must be high. In experiments on human subjects, for example, McCullough & Eisele (1951) and Hormaeche, Peluffo & Aleppo (1936) have shown that the infection dose for *Salm. anatum*, *Salm. malagridis* and *Salm. typhi-murium* must amount, in the majority of cases, to several millions of bacteria. Hormaeche, Peluffo & Aleppo (1940) have pointed out that the infection dose for children is lower than for adults. It is thus reasonable to assume that for infants the infection dose is considerably lower. The present investigation supports the assumption that even very low doses of *Salm. muenchen* may be sufficient to produce a serious infection in infants.

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THE STERILIZATION OF GOAT SKIN WITH ETHYLENE OXIDE AND SOME BACTERIOLOGICAL STUDIES OF THE PROCESS

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SUMMARY: Because it is uncertain whether the usual methods of sterilizing leave the normal structural characteristics of hide and skin unaltered, studies are usually made on hide powder and the results so obtained extended to hide and skin. The results of such studies, however, sometimes prove confusing and the conclusions drawn may be false.

In the present investigation skins were so treated with ethylene oxide as to render them bacteriologically sterile and the influence of any residual effect on the activities of bacteria subsequently cultured on the skins was studied from different aspects.

A basic question of great importance in the microbiological study of hides and skins is whether they can be so treated as to make them bacteriologically sterile and yet retain practically all the characteristics of a normal skin. Only then would it be possible to study the action of pure cultures of bacteria on hides and skins. The literature shows that occasional attempts have been made in this direction, but only with a view to obtaining better preservation (Anderson, 1949), and in none of them was true bacteriological sterility obtained. Due to lack of knowledge on the influence of sterilizing methods on the properties and structure of hides and skins, bacteriological investigations have so far been carried out with hide powder sterilized by dry heat at 100° for 1 hr and the results obtained have been used to deduce the reactions expected with hide and skin; but the conclusions reached have sometimes been confusing (Oakley, Warrack & van Heyningen, 1946).

Evans (1947) studied the production of collagenase by certain species of *Clostridium*, tested the enzyme on hide powder and subsequently extended the results so obtained to natural hides and skins. Oakley, Warrack & Warren (1948) were of the opinion that hide powder was not a reliable indicator for collagenase because during the preparation of the powder the collagen was so altered as to make it more susceptible to attack by the specific bacterial enzyme. Hide powder was therefore not considered to be a reliable indicator for collagenase, though it could provide reasonable presumptive evidence that a collagenase was present. Oakley & Warrack (1950) later pointed out the risk involved in extending results obtained with hide powder to hide and skin by demonstrating that *Cl. histolyticum* had two specific enzymes, one which attacked both collagen and hide powder and the other which attacked hide powder but not collagen. This shows the importance of using hide and skin and not hide powder for any specific test for collagenase and indicates the imperative need for a method of sterilizing hides and skins which would cause the minimum of denaturation. The necessity is still more obvious when it is required to test hide and skin from which the hair has not been removed.

In recent years, ethylene oxide (1,2-epoxide) has been used as a fumigating agent for preserving a number of food materials and as a disinfecting and sterilizing agent for glass and metal surfaces, blankets, linens, etc. (Baerwald, 1945; Verhoeven, 1947; Dyson, 1950; Royce & Sykes, 1955). Whereas different 1,2-epoxides are known to react to different extents with certain groups in proteins (Fraenkel-Conrat, 1944), Alexander, Carter, Earland & Ford (1951) showed that of several 1,2-epoxides tested, ethylene oxide was the least reactive and that in the anhydrous state the reaction was still less. Working with hair and wool they found that even a 5% aqueous solution of ethylene oxide reacted only slightly. In view of these findings, Noval & Nickerson (1959) treated hair and wool with ethylene oxide and claimed that bacteriological sterility was achieved: but, as not much is known about the reaction of ethylene oxide with hide and skin *vis-a-vis* its sterilizing effect, this investigation was therefore undertaken.

EXPERIMENTS AND RESULTS

Sterilization procedure

A piece of fresh goat skin was thoroughly washed with water, hung up until air-dry and then cut into strips about 1 in. long and 0·25–0·5 in. wide. These were placed in Petri dishes which were put in empty desiccators. Large pieces of skin were also included to see if they behaved differently. These pieces were also intended to be made into leather and compared with that produced from untreated skin, but the results of these tests are not given in the present paper.

Well cooled ethylene oxide (B.D.H.) was put into the desiccators and the lids quickly replaced and held tight by mechanical means. This was necessary because of the high volatility of ethylene oxide (B. Pt. about 10·5°) and to prevent the gas from escaping. The amounts of ethylene oxide used were 0·5, 1·0, 2·0 and 3·0 ml/litre of space in the desiccators and the treatment times were 8, 12, 16, 20 and 24 hr at room temperature (about 30°) at each dose level. After the appropriate times, the desiccators were opened with aseptic precautions and the strips of skin tested for sterility by putting them either into nutrient broth to reveal aerobes or cooked meat medium to reveal anaerobes, incubation being at 37° in both cases. The nutrient broth was examined after 48 and 96 hr and the cooked meat after 7 days. From the extent of growth it was concluded that fumigation with 0·5 ml/litre and upwards was effective provided that exposure was for not less than 12 hr. Accordingly, in all subsequent experiments, a dose of ethylene oxide of 1 ml/litre of the fumigating space and a reaction period of 12 hr were employed. This resulted in 70–80% of the treated skin pieces being sterile when tested by aerobic incubation; when tested by anaerobic incubation the percentage was still higher. The percentage of sterility could be still further increased by increasing the dose of ethylene oxide. However, this resulted in loosening of hair, and the lower dose was therefore not exceeded. From the larger pieces of skin, several hairs from different places were pulled out aseptically and tested for sterility; several areas on the flesh side were also swabbed and cultured. The results of tests showed that the large pieces were also sterilized by the same treatment as the small strips.

Effects of sterilization on the skin

Since it was intended to test, in later work, the susceptibility of the sterilized skin to bacterial attack, it was necessary to know if the treatment had left any after effects which could interfere with these tests. It was envisaged that the treated skin might contain residual ethylene oxide, which in turn might exert an antibacterial or possibly even a mutagenic effect. Furthermore, the skin might contain newly formed compounds of an antibacterial or stimulatory nature.

Growth inhibitory and mutagenic effects. The possible growth inhibitory and mutagenic effects of treated skin on vegetative cells of *Bacillus subtilis* were examined first. Strips of treated skin were placed in tubes of 1% peptone broth and these, and similar broth tubes without added skin, were inoculated with *B. subtilis*. After 18 hr incubation at 37° subcultures from both were made into media to determine the behaviour of the organism when provided with various carbohydrates (glucose, lactose, sucrose, maltose, mannitol and xylose) and its behaviour in the indole, methyl red and Voges-Proskauer tests. The time taken for the first appearance of visible growth in nutrient broth was also noted. In no case did the culture grown in the presence of treated skin exhibit any difference from the culture grown in its absence, and although tests of this sort are of very limited value they do indicate that the treated skin had exerted no major action on the organism. However, it was decided to investigate the matter further, to see if treated skin could influence either the germination or sporulation of *B. subtilis*, since both these processes are known to be affected by inhibitory and stimulatory substances.

Effects on spore germination. A culture of *B. subtilis* on peptone agar was incubated at 30° for 8–10 days, when sporulation was virtually complete. The spores were washed from the culture with sterile normal saline and washed twice by centrifuging. The sediment was resuspended in normal saline and heated at 80° for 30 min to kill remaining vegetative cells. According to Hashimoto, Black & Gerhardt (1960) this treatment kills all forms except mature spores. The suspension was then diluted with sterile normal saline until the viable count as determined by plating was $2\cdot5$–$3\cdot0 \times 10^6$/ml. Tubes containing 5 ml of 1% peptone water, with and without strips of sterilized skin, were inoculated with 0·2 ml of spore suspension and incubated at 37°. At short intervals samples of 0·02 ml were withdrawn, spread over areas of 22 mm^2 on microscope slides, fixed and stained with methylene blue as described by Powell (1950). On each slide 100 random fields were observed and the numbers of vegetative cells counted. It was seen that, independent of the presence of sterilized skin, no germination occurred for 3½ hr. After this time vegetative cells were seen and they became more numerous as time proceeded, but they were equally numerous in preparations from cultures with and without skin and no inhibitory or stimulatory effects were observed. The counts of vegetative cells/field were: control (no skin), 61, 88 and 420; and in the presence of sterilized skin, 94, 252 and 437, after 4, 5 and 6 hr respectively.

Effects on spore formation. Wynne (1952) observed that the formation of spores was not directly a consequence of prolific vegetative development but was a secondary process which may or may not take place, and which was very sensitive to many physiological conditions. It can be inhibited in circumstances in which vegetative growth is completely unaffected and is, in fact, the more exacting process.

To determine whether treated skin contained material which would affect sporulation, *B. subtilis* was inoculated into tubes containing 5 ml of 1% peptone water with and without strips of sterilized skin. Quantities of 0·1 ml were removed at intervals of 24 hr during incubation at 37°, heated at 80° for 30 min to kill vegetative cells and plated (after suitable dilution in sterile saline) on 2% peptone agar to obtain the numbers of spores. The inoculum for the cultures consisted of 1 loopful of a 6 hr 1% peptone broth culture, which itself had been seeded from a similar culture and could therefore be safely assumed to be free from spores.

The results (Table 1) show that the presence of sterilized skin made no difference to the rate of sporulation.

Table 1. *A comparison of the rates of sporulation of* B. subtilis *in 1% peptone water with and without sterilized goat skin*

Incubation period (hr)	Spore count (10^6/ml) with skin	
	Absent	Present
24	1·4	0·8
48	62	67
72	1260	1150

DISCUSSION

It has been shown above that ethylene oxide can be used to sterilize air-dry goat skin. The skin was dried before treatment both because ethylene oxide is known to react less extensively with proteins when the moisture content is small, and because without drying partial hair slip might occur after the treatment.

It is known (Rapoport, 1948; Kolmark & Westergaard, 1953) that ethylene oxide has mutagenic properties and it was therefore possible that ethylene oxide treated skin might induce mutations in bacteria cultivated in its presence. The tests made, however, did not reveal any such effect and although the matter was not pursued to the extent of determining mutation rates in the presence and absence of sterilized skin, it is considered unlikely that sufficient of the chemical remained for there to have been any such effect.

The examination of the ability of treated skin to affect spore germination and spore formation also revealed no obvious effect, but it must be remembered that the tests were done in the presence of peptone and this may have masked any slight stimulatory or inhibitory properties possessed by the treated skin. Bacteria growing on treated skin in the absence of added nutrients might behave differently. However, the indications that the ethylene oxide treatment had not significantly altered the skins are supported by the finding (Sen *et al.*, unpublished data) that leather made from them did not differ in quality from that made from untreated skin.

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THE MICROFLORA OF THE RUMEN OF THE YOUNG CALF: II. SOURCE, NATURE AND DEVELOPMENT

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SUMMARY: Twelve calves of different sex and breed divided into four groups were used in the experiments. Each group received different rearing and diet treatment. Three hundred and nine samples of rumen contents were obtained by means of a stomach tube during the period from birth to three months of age. Streptococci, lactobacilli and coli-aerogenes organisms were isolated and studied, and isolates were also made from various elements of the calves' environment.

Rapid development of the rumen flora occurred immediately after birth of the animals. During the first week of life, streptococci and coli-aerogenes organisms predominated, whereas no lactobacilli were isolated from calves on any of the treatments. The coli-aerogenes count (10^7–10^8/g wet weight) was higher than in the adult rumen, but dropped to its normal level during the succeeding two weeks. The streptococcus and lactobacillus counts varied according to treatment.

Streptococcus bovis was the predominating streptococcal species, except during the first week of life, when *Strep. faecium* predominated and *Strep. bovis* amounted to only 28·6% of the total streptococcal isolates. The variation in physiological characteristics within the *Strep. bovis* strains was greater than normally found within strains from adult cattle. *Lactobacillus acidophilus*, *L. plantarum* and a group of strains which resembled *L. brevis* predominated among the lactobacilli. Most of the coli-aerogenes organisms were *Escherichia coli*, faecal type I.

The diet was found to influence the proportion of streptococci and lactobacilli in the rumen, whereas the incidence of various species of the two groups of bacteria, particularly the streptococci, was associated with the age of the animals.

The microflora of the environment influenced the rumen flora only during its early development, soon after birth of the calves.

The development of the rumen flora of the calf is of interest from both physiological and economic points of view. A knowledge of the extent and type of fermentation in the rumen of the young calf may be of value in understanding the process of adaptation to the adult form of ruminant metabolism, i.e. to the utilization of large amounts of volatile fatty acids, a main end product of bacterial fermentation in the rumen. The early development of rumen function and, consequently, the possibility of early feeding of roughages may reduce costs of calf rearing.

Pounden & Hibbs (1948*a*,*b*) studied the establishment of certain micro-organisms in the rumen of young calves using direct microscopic methods and divided the organisms into 2 groups associated with rations consisting predominantly of hay or of grain. They found that the rumen contents of young calves contained few characteristic rumen organisms and that inoculation with rumen contents from adult animals hastened establishment of organisms associated with hay diets.

Huhtanen, Saunders & Gall (1951) studied some 200 calves from 8 different localities and found 9 organisms not normally present in the rumen of adult animals and 11 common to both young and adult animals. The most common type were fast growing lactic acid bacteria. However, subsequent studies (Huhtanen & Gall, 1953*a*,*b*) revealed that bacteria typical for young animals could also be found in adults on a high concentrates diet.

Workers at the Rowett Research Institute (MacKay & Oxford, 1954; Mann, Masson & Oxford, 1954*b*; Mann & Oxford, 1954, 1955) have described several organisms isolated from the rumen of young calves and kids. They included lactobacilli, streptococci, coli-aerogenes organisms and Gram-negative, facultatively anaerobic, urease positive rods. Recently Hobson, Mann & Oxford (1958) described a large, Gram-negative, anaerobic lactate fermenting coccus. It was found in large numbers (10^9/ml) in the rumen of young calves, and only rarely and in small numbers in the rumen of adult cattle and sheep.

Bryant, Small, Bouma & Robinson (1958) studied the microflora of 3 calves between 1 and 13 weeks of age; samples were taken at intervals of 3 weeks. The total count of anaerobic bacteria was slightly higher than in adult animals, as was the count of lactate fermenting bacteria, facultative anaerobes and coli-aerogenes organisms. All these counts decreased with age. An estimate of the cellulolytic bacteria showed that they could be found in relatively large numbers when the calf was only 1 week old; in calves 3 weeks of age their presence was quantitatively comparable to that in the adult animal.

Eadie, Hobson & Mann (1959) studied the rumen organisms of 2 young calves, each fed a different diet. The calves were sampled at weekly intervals and at the same time inoculated with a heavy suspension of rumen ciliate protozoa, both by means of a stomach tube. The establishment of protozoa was studied and counts of lactobacilli, a lactate utilizing coccus (LUC) and *Veillonella gazogenes* determined. The calf fed a diet rich in concentrates (1,800 g/day) had high lactobacilli and LUC counts and a lower ruminal pH. The protozoa in this animal became established only after 12 weeks. The calf fed a ration consisting predominantly of hay had, after the initial period of milk feeding, very low lactobacilli and LUC counts. The pH of the rumen fluid was higher and rumen ciliate protozoa were present throughout the experimental period. The *Veillonella* counts were the same in both calves.

More information is necessary to appreciate the role and importance of various groups of bacteria in the development of rumen function. The present study was made to obtain information on the source, nature and development of some of the more numerous groups of bacteria in the rumen of the young calf.

MATERIALS AND METHODS

General

Animals and treatments. Twelve calves of different sex and breed were used as experimental animals, and were kept from birth until 3 months of age. They were divided into 4 groups, each group receiving a different treatment.

Treatment 1 was a conventional calf rearing method. The animals were allowed to suckle their dams for 4 days, after which they were penned separately for bucket feeding. They received 1 gallon of whole milk daily for the next 3 weeks, good quality hay being offered. After 3 weeks, milk was replaced by a milk substitute of the following composition: dried skimmed milk, 74 parts; oat flour, 20 parts; Dextrolac (Corn Products Co. Ltd., London), 5 parts; Drivite (vitamins A+D, Boots Pure Drug Co. Ltd., Nottingham), 1 part. Water (12 lb) was added to $1\frac{1}{2}$ lb of the mixture which was fed daily in two parts. In addition to milk substitute and hay, concentrates were fed to appetite, to a maximum of 3 lb/day, in the form of the following mixture: linseed cake, 4 parts; dredge corn, 4 parts; flaked maize, 4 parts; bran, 2 parts; fish meal, 1 part; cod liver oil, 4 oz/cwt.

Treatment 2 was the same as treatment 1, but the calves were allowed to suckle their dams for 1 day only.

In treatment 3 the calves suckled their dams for 4 days, and then suckled a nurse cow brought in twice daily, throughout the experimental period. Good quality hay was also offered and the calves had access to the concentrates fed to the nurse cow.

In treatment 4 the calves suckled their dams for 4 days and were then penned separately and bucket fed. After an initial period (3 weeks) of whole milk feeding, solid food only was given in the form of a special concentrates mixture consisting of: flaked maize, 40 parts; dredge corn, 20 parts; linseed meal, 14 parts; dried skimmed milk, 10 parts; molassine (dried peat moss meal soaked in molasses), 10 parts; fish meal, 5 parts; vitamins (Drivite), 1 part; Aurofac 2A (Cyanamid of Great Britain, Ltd.) was added to the dry feed to allow a maximum possible intake of 50 mg of chlortetracycline/day. Although Aurofac was incorporated in the diet, it was not the intention of the work to study specifically the effect of the antibiotic on the development of the rumen flora. The animals were fed to appetite to a maximum of 5 lb/day, good quality hay also being offered.

Sampling. Rumen samples were taken by means of a stomach tube. The calves were sampled, starting on the first or second day after birth, every second day during the first 10 days and subsequently twice a week. Volumes of 20–25 ml of rumen contents were withdrawn into 250 ml conical flasks, no special anaerobic precautions being observed. In addition to bacteriological examination (see later) pH measurements were made of this material.

Samples were also taken from potential sources of micro-organisms in the calves' environment: from the bedding and the air of calving boxes and calf pens, from the dams' rumens using the cud sampling technique described by Davey & Briggs (1959), from the dams' colostrum and milk, and from the whole milk, milk substitute and solid food fed by bucket. Air samples were taken, by means of a slit sampler, directly on to the surface of reinforced clostridial medium (RCM) and MacConkey's agar plates exposed for 15 min.

Microbiological methods

Media. Details of the media and technique used for viable counts are given by Wilson & Briggs (1955). MacConkey's broth was used for the enumeration of coli-aerogenes bacteria, a separate dilution series in physiological saline being made for this purpose as it was found that the use of RCM as diluent tended to diminish selectivity. Five tubes of RCM and 5 tubes of MacConkey's broth were used at each dilution level above 10^{-6} and 10^{-2}, respectively.

Isolations, which were made from the highest positive dilution tubes, were either freeze dried or maintained in a refrigerator at 4°. Gram-positive, catalase negative cocci were regarded as presumptive streptococci and Gram-positive, catalase negative, nonsporing rods as presumptive lactobacilli.

Streptococci. The physiological and serological tests used for characterization of the streptococci were those of Perry, Wilson, Newland & Briggs (1955) with the addition of tests for sensitivity to 1:2,500 potassium tellurite, and the use of concentrated HCl extracts (Shattock, 1949) in precipitin tests.

Lactobacilli. The physiological tests used for characterization of the lactobacilli were those given by Perry & Briggs (1957).

Coli-aerogenes organisms. These were examined by IMViC and Eijkman tests.

Protozoa. Crude rumen material from the calves was examined microscopically and estimates of the numbers of protozoa were made.

RESULTS

Animals and treatments

The live weight gains and general development of the calves were satisfactory on all treatments. Calves on treatment 3 (suckling their dams and a nurse cow) grew best, as might be expected, but no differences among the remaining three groups were observed. Calves on treatment 4 scoured slightly for a few days when the milk diet was stopped. At the end of the experimental period one calf from each group was slaughtered and the stomachs examined; no marked differences in the development of the compartments or of the mucosae were observed.

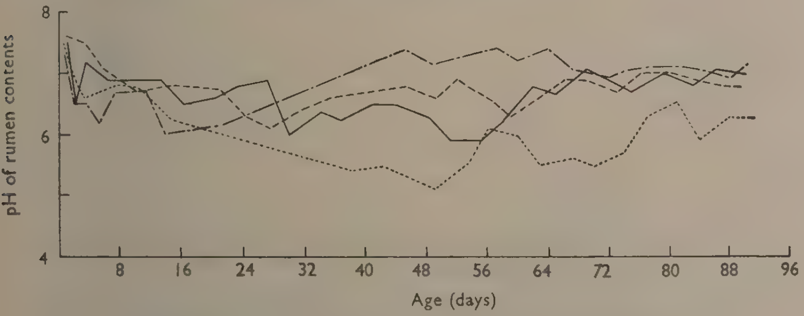

Fig. 1. Changes with age in the pH of the rumen contents of calves on four different treatments. Treatments: 1, continuous lines; 2, dashes; 3, chain dots; 4, dots.

The rumen fluid taken a few hours after birth was a rather thick, slimy, opalescent fluid, containing small amounts of hair and meconium. The pH was rather high (7·5–7·6) but dropped on the next day to below 7·0. As can be seen in Fig. 1, the pH values of the rumen contents differed according to the treatment.

Bacteriology

The bacterial picture of the rumen contents during the first week of life was similar in all the calves. On the first day the rumen fluid contained only a small number of organisms, but they increased rapidly to reach, on the second day, the numbers normally found in the adult animal, or even slightly more. Streptococci and coli-aerogenes organisms predominated and lactobacilli were not isolated during the first week of life. The coli-aerogenes count (10^6–10^7/g wet weight) was many times higher than that normally found in the adult, and at the end of the first week started to decrease to reach, during the third week, its normal level of 10^3–10^4/g. The numbers of streptococci and lactobacilli varied according to the treatment.

With treatment 1 the streptococcus count increased rapidly during the first day, reaching 10^8/g on the second day after birth (Fig. 2). At the end of the second week the numbers of streptococci began to diminish and lactobacilli appeared. The numbers of lactobacilli soon exceeded those of the streptococci, remaining subsequently at the 10^8–10^9/g level. After the milk was replaced by milk substitute a further slight increase in the lactobacilli followed, with a corresponding decrease in the numbers of streptococci, the difference between the two groups being then of the order of 10^3. After about 10 weeks this difference tended to diminish again. The pH changes (Fig. 1) were to some extent related to the changes in bacterial counts, particularly those of the lactobacilli.

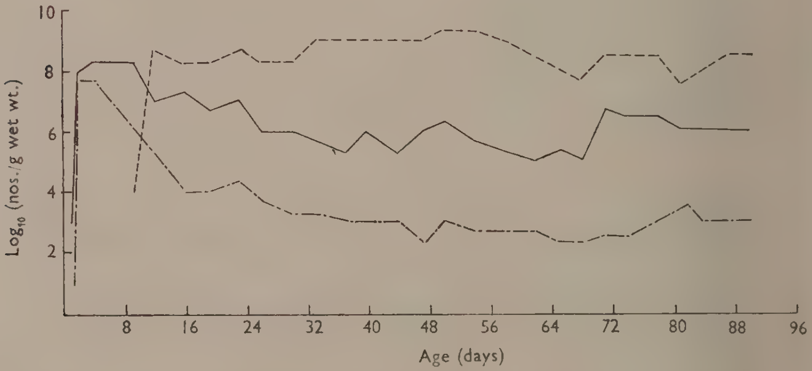

Fig. 2. Changes with age in the rumen population of streptococci (continuous lines), lactobacilli (dashes) and coli-aerogenes organisms (chain dots) in calves on treatment 1.

The behaviour of the lactic acid flora in treatment 2 was similar to that in treatment 1. The initial rapid increase in the numbers of streptococci was followed after one week by a decrease, and the appearance of lactobacilli, which then predominated during the following 4 weeks (Fig. 3). The difference between the two groups subsequently diminished and they predominated in turn, the pH of the rumen contents changing correspondingly.

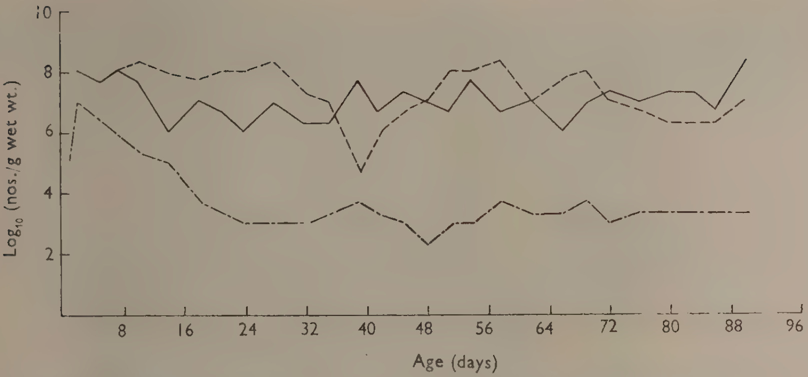

Fig. 3. Changes with age in the rumen population of streptococci (continuous lines), lactobacilli (dashes) and coli-aerogenes organisms (chain dots) in calves on treatment 2.

With treatment 3 the initial high streptococcus count (10^8–10^9/g) decreased only slightly (to 10^7/g), the streptococci predominating throughout the experimental period (Fig. 4). Lactobacilli were isolated only occasionally. The pH of the rumen fluid, beginning from the second month, was higher than in calves on other treatments. The calves on this treatment were the only ones to develop a fairly rich protozoal fauna.

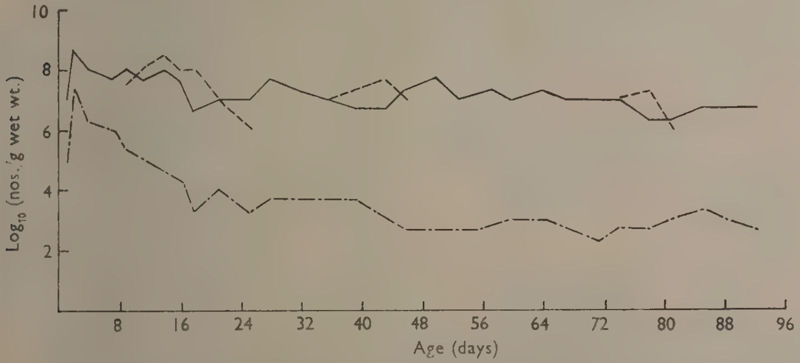

Fig. 4. Changes with age in the rumen population of streptococci (continuous lines), lactobacilli (dashes) and coli-aerogenes organisms (chain dots) in calves on treatment 3.

With treatment 4, streptococci predominated during the first week (Fig. 5). Lactobacilli then appeared, their numbers soon reaching 10^8–10^9/g, remaining at that level until the end of the experimental period. The streptococcus count decreased as the lactobacilli appeared, and there was a further drop after the change to the milk free, solid concentrate diet. The pH of the rumen fluid, which was similar to that of the other groups during the first three weeks, dropped subsequently to 5·2 5·5; it tended to increase again during the third month but, even so, was the lowest of any treatment throughout the experimental period.

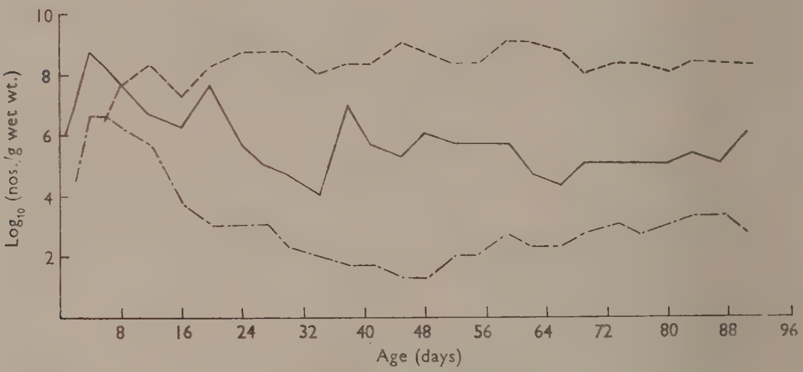

Fig. 5. Changes with age in the rumen population of streptococci (continuous lines), lactobacilli (dashes) and coli-aerogenes organisms (chain dots) in calves on treatment 4.

Bacteria from the environment. These were mainly common saprophytes, micrococci of various types predominating. Sporeforming rods, pseudomonads and coli-aerogenes organisms were often found, together with fewer streptococci and lactobacilli. The numbers and species of streptococci, lactobacilli and coli-aerogenes organisms found in various elements of the environment are shown in Table 1.

Table 1. *The occurrence of various species of streptococci, lactobacilli and coli-aerogenes organisms in elements of calves' environment*

Genus or group and no. examined		Identification	Occurrence* of strains in					Total no. examined
			Air	Bedding	Food	Colostrum	Dam's rumen	
Streptococcus (58)	Group D	*Strep. bovis*	+	+	+	−	+	25
		Strep. faecium	+	+	+	−	−	13
		Strep. faecalis	+	−	+	−	−	3
		Strep. faecalis var. *zymogenes*	−	+	−	−	−	3
		Strep. durans	+	+	+	−	−	5
	Group B		+	+	−	−	−	2
	Group C		−	−	−	+	−	2
	Group E		−	−	+	+	−	4
	Unclassified		+	−	−	−	−	1
Lactobacillus (18)		*L. acidophilus*	−	+	+	−	+	6
		L. plantarum	+	+	−	−	+	6
		Lactobacillus spp.†	−	−	+	−	+	6
Coli-aerogenes bacteria (23)		*E. coli*	−	+	+	−	+	23

* +, present; −, absent. † See text.

Strep. bovis and *Strep. faecium* were the streptococci most frequently found. Other species and lactobacilli were only occasionally isolated; nevertheless, they were present in the calves' environment in detectable numbers.

Table 2. *The classification of 247 strains of streptococci from the rumen of calves*

Serological group	Species	No. of strains
D	*Strep. bovis*	204
	Strep. faecium	21
	Strep. faecalis	11
	Strep. faecalis var. *zymogenes*	1
	Strep. durans	6
B		1
E		1
G		1
Unclassified		1

Streptococci. The classification of 247 strains of streptococci from the rumen of calves is shown in Table 2, and the physiological characteristics of group D strains isolated from the rumen and environment of the calves are given in Table 3.

Table 3. *The biochemical and physiological characteristics* of 292 strains of group D streptococci isolated from the rumens and environments of calves*

Test	Identity and no. of cultures examined							
	Strep. bovis, types				*Strep. faecium*	*Strep. faecalis*	*Strep. faecalis* var. *zymogenes*	*Strep. durans*
	I (146)	II (40)	III (31)	IV (12)	(34)	(14)	(4)	(11)
Fermentation of:								
Arabinose	—	—	+	—	+	—	—	—
Glycerol	—	—	—	—	— (55·9)	+	+	—
Mannitol	—	—	—	+	+	+	+	—
Raffinose	+	+	+	+	—	—	—	—
Action in litmus milk	—	ACR	ACR	ACR	ARC	ARC	ARC	ACR
Gelatin liquefaction	—	—	—	—	—	—	+	—
Hydrolysis of starch	+ (98·6)	— (65·0)	+	+	—	—	—	—
Hydrolysis of arginine	—	—	—	—	+	+	+	+
Growth at 45°	+	+	+	+	+	+	+	+
Growth in 6·5% NaCl	—	—	—	—	+	+	+	+
Survival at 60° for 30 min	— (75·3)	+ (52·5)	— (87·1)	—	+	+	+	+
Resistance to potassium tellurite	—	—	—	—	—	+	+	—
Haemolysis in 5% horse blood	α	α	α	α	α to γ	α to γ	β	γ

* +, positive reaction; —, no reaction; A, acid; C, clot; R, reduction. Numbers in brackets show the percentages of strains giving the reaction indicated.

The 229 strains identified as *Strep. bovis* differed with regard to some physiological characteristics, the differences forming the basis of certain groups, e.g. strains which did not ferment arabinose or mannitol and did not change litmus milk, but fermented starch; strains which fermented neither arabinose nor mannitol, but which changed the litmus milk and did not normally ferment starch; strains which fermented either arabinose or mannitol and also invariably changed litmus milk and fermented starch.

All the *Strep. bovis* strains have been grouped into 4 physiological types on these reactions. The incidence of these types in the rumen was related to the age of calves, a relationship which is discussed later.

Other species of streptococci were fairly uniform with regard to physiological characteristics, except for glycerol fermentation by *Strep. faecium* (55·9% of the strains were negative, the remainder positive). There was no difference in the proportion of glycerol fermenting to glycerol nonfermenting strains between isolates from the rumen and the environment of the calves.

Some doubt exists as to the identification of *Strep. durans* in this study, as none of the 11 strains isolated showed β-haemolysis, which is one of the distinctive characters of the species. All the other physiological characteristics corresponded with those of *Strep. durans.*

Table 4. *The incidence of various species of streptococci, isolated from the rumen of calves, in relation to the age of the animals*

Serological group	Species	Incidence* (% of total) for calves aged (weeks)												
		1	2	3	4	5	6	7	8	9	10	11	12	13
D	*Strep. bovis*	28·6	84·7	80·0	85·7	83·4	81·9	100·0	90·0	100·0	92·5	86·2	91·7	90·5
	Strep. faecium	35·8	15·3	10·0	14·3	16·6	13·6					4·6	4·2	
	Strep. faecalis	25·0		10·0							3·8	4·6		
	Strep. faecalis var. *zymogenes*												4·1	
	Strep. durans						4·5		5·0		3·7	4·6		9·5
B		3·6												
E		3·5												
G		3·5												
Unclassified							5·0							

* For clarity of presentation zeros have been omitted.

The diet appeared to have had no effect on the composition of the rumen streptococci, but a marked relationship existed between these organisms and the age of the calves, as shown in Table 4. The relationship was similar in all groups, irrespective of diet. During the first week of life the streptococcus population in the rumen consisted of several species; *Strep. bovis* constituted only 28·6% of the total number of isolated strains, whereas *Strep. faecium* predominated. Of 42 strains of streptococci other than *Strep. bovis* isolated from the rumen, 22 were from calves less than one week old. During the second week *Strep. bovis* became the predominating species (84·7%). After some 6 weeks *Strep. bovis* constituted over 90% of all the streptococci isolated. In 5 calves (2 from treatment 2 and 1 each from treatments 1, 3 and 4) *Strep. bovis* was the predominating species from the first day of life.

A marked relationship was observed between the age of calves and the incidence in their rumens of the various physiological types of *Strep. bovis* mentioned previously (Table 5). This relationship was evident in calves on all treatments. Types I and III were characteristic of older calves. Type I was also found during the early development of the calves but only predominated from the fifth week. Type III was not isolated during the first 3 weeks, but was subsequently isolated regularly to constitute from 6·2 to 21·4% of the total number of *Strep. bovis* strains. From the rumens of the dams, 10 out of 13 *Strep. bovis* strains isolated were type I, and 3 type III. Type II predominated during the first 4 weeks of life and then decreased. Type III was only occasionally found and no relationship was observed between this group and any other factor studied. In the calves' environment all 4 types were found in the following proportions: type I, 5 strains; type II, 4 strains; type III, 1 strain; type IV, 2 strains.

Table 5. *The distribution of various types of* Strep. bovis *in the rumen of calves in relation to age*

Type	Incidence* (% of total) for calves aged (weeks)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
I	41·8	36·4	50·0	38·4	63·6	70·6	64·3	73·4	87·6	66·7	84·2	68·2	76·5
II	50·0	63·6	37·5	46·2	18·2	23·5	14·3	13·3	6·2	4·1		9·1	
III				7·7	9·1		21·4	13·3	6·2	20·8	15·8	13·6	17·6
IV	8·2		12·5	7·7	9·1	5·9				8·4		9·1	5·9

* For clarity of presentation zeros have been omitted.

Lactobacilli. The 160 strains isolated from the rumen were grouped into the following species: *L. acidophilus* (86), *L. plantarum* (19), *L. fermenti* (2), *L. casei* (1)

Table 6. *The biochemical and physiological characteristics* of* 171 *strains of lactobacilli isolated from the rumens and environments of calves*

Test	Identity and no. of cultures examined				
	L. acidophilus (92)	*L. plantarum* (25)	*Lactobacillus* spp.† (47)	*L. fermenti* (2)	'Intermediate' (5)
Fermentation of:					
Amygdalin	+	+	−	−	±
Arabinose	− (87·0)	+	+	+	+
Cellobiose	+ (84·8)	+	−	−	±
Glycerol	−	− (88·0)	−	−	−
Inositol	−	−	−	−	−
Lactose	+	+	−	+	+
Mannitol	−	+ (55·0)	−	−	−
Melezitose	−	−	−	−	−
Melibiose	+ (95·7)	+	− (55·3)	+	+
Raffinose	+ (84·8)	+	−	+	+
Salicin	+ (78·3)	+	−	−	+
Sorbose	−	− (92·0)	+	−	−
Sucrose	+ (92·4)	+	−	+	+
Trehalose	− (58·7)	+	−	−	−
Xylose	− (94·6)	− (88·0)	+	+	−
Gas from glucose	−	−	−	+	−
Ammonia from arginine	−	−	−	+	+
Growth at 15°	−	+	−	−	−
Growth at 45°	+	−	−	+	+

* +, positive reaction; −, negative reaction. The numbers in brackets below the reactions show the percentages of strains giving the reactions indicated. † See text.

Table 7. *The incidence of various species of lactobacilli, isolated from the the rumen of calves, in relation to the age of the animals*

Identification	Incidence* (% of total) for calves aged (weeks)												
	1	2	3	4	5	6	7	8	9	10	11	12	13
L. acidophilus		33·2		33·3	44·5	61·5	57·2	73·6	47·5	63·2	55·0	61·1	42·9
L. plantarum		50·2	66·7	11·1	22·2	7·7		19·8	10·5	5·2	15·0	33·3	7·1
Lactobacillus spp.†		16·6		44·5	22·2	23·1	35·7	6·6	26·3	31·6	10·0		42·9
L. fermenti											5·0	5·6	
L. lactis											5·0		
Unclassified			33·3	11·1	11·1	7·7	7·1		15·7		10·0		7·1

* For clarity of presentation zeros have been omitted. † See text.

and *L. lactis* (1). A group of 41 presumptive lactobacilli that resembled *L. brevis* physiologically, but which could not be identified with certainty, has been designated *Lactobacillus* spp. in Tables 1, 6 and 7. Of the remaining 10 unidentified strains, 5 resembled those isolated from the pig by Fewins, Newland & Briggs (1957) and described as 'intermediate lactobacilli' in view of their general resemblance to the heterofermentative *L. fermenti* while lacking the property of gas production from glucose. The physiological characteristics of the lactobacilli isolated from the rumens and the environment of the calves are shown in Table 6. The relationship between the age of calves and the composition of the lactobacillus population in the rumen was not so well marked as with the streptococci. During the first week of life no lactobacilli were found. During the second and third weeks *L. plantarum* was the predominant species, after which *L. acidophilus* predominated (Table 7). No relationship was found between the incidence of the various species of lactobacilli and the composition of the diet. The three most frequently isolated species were found in calves on all treatments.

Discussion

The rumen flora of all the calves developed rapidly immediately after birth, reaching on the second day a quantitative level similar to, or higher than, that found in the adult rumen.

The first organisms that developed in the new-born calves on all treatments were streptococci and coli-aerogenes bacteria. The numbers of the latter were particularly high during the first week and then decreased gradually to reach, after some 3 weeks, the level normally found in adult cattle. This seems to be a general feature, having been observed by Ziolecki (1958) in Poland and Bryant *et al.* (1958) in America. The reason for the decrease was in all probability the increasing competition from other groups of bacteria. *E. coli* faecal type I was the predominant type isolated, together with a few strains of Intermediate type I. No strains of *Klebsiella aerogenes* were isolated.

Lactic acid bacteria are undoubtedly important constituents, at least numerically, of the rumen flora of calves. They were present at all times in large numbers, streptococci and lactobacilli being the main representatives, with a complementary relationship existing between the two genera; an increase in the numbers of one genus was normally associated with a decrease of the other. Thus, the lactic acid producing population in the rumen remained fairly constant, but with varying proportions of its constituents. A marked influence on these proportions was exerted by the type of diet. An increased consumption of concentrates invariably caused an increase in the numbers of lactobacilli and reduced the numbers of streptococci, whereas increased hay consumption stimulated the streptococci and depressed the lactobacilli; similar observations had been made previously by Perry *et al.* (1955) in adult animals. Calves on treatment 4, which received the largest amounts of concentrates, had rumen contents having the highest lactobacillus and the lowest streptococcus counts, and also the lowest pH. The difference in the numbers of lactobacilli between calves on this treatment and those on treatments 1 and 2, which also received a diet rich in concentrates, was not large, but a marked difference was observed between the pH values of the rumen contents. The explanation for this finding may be that calves on treatments 1 and 2 received a substantial part of the ration in the form of milk substitute, i.e. in the form of a fluid which would pass directly into the abomasum. Calves on treatment 4 were fed exclusively on dry, solid food which must have entered the rumen, increasing there the amount of readily fermentable carbohydrates; this may have stimulated the activity of lactobacilli and thus depressed the pH. A low pH value of the rumen contents has been found to affect adversely the development of other rumen bacteria, particularly the cellulolytic flora (Hungate, Dougherty, Bryant & Cello, 1952); thus, from the bacteriological point of view, this treatment seems to have certain disadvantages.

Calves on treatment 3 were fed milk and hay alone, though they had access to the concentrates fed to the nurse cow; lactobacilli were isolated only occasionally and streptococci predominated. After the initial few weeks, the pH of the rumen contents was the highest of all treatments. In treatments 1 and 2 the differences between the numbers of streptococci and lactobacilli were much smaller. These differences in treatments 1, 2 and 4 tended to diminish towards the end of the experiment, the pH values increasing concurrently. The reason for this was probably that the food consumption of the calves increased. However, since the amount of concentrates in the ration was limited, hay consumption increased, thus diminishing the relative proportion of concentrates in the diet; this was reflected in higher pH values and counts of streptococci.

Only the calves on treatment 3 developed a fairly rich protozoal fauna. This was probably the result of the high pH values of the rumen fluid of calves in this group which allowed the early development of protozoa. This group of calves alone had direct contact with adult animals after the first few days of life, which may well have predisposed towards the development of the rumen fauna, although the recent work of Eadie, Hobson & Mann (1959) suggests also that the composition of the diet and the resulting pH of the rumen contents influence the establishment of protozoa in the rumen of calves.

The Aurofac (chlortetracycline) supplement in treatment 4 appeared to have no influence on the components of the microflora studied despite the fact that it was incorporated in dry food and must therefore have entered the rumen. The small numbers of streptococci, high numbers of lactobacilli and low pH were thought to be the results of the diet rather than of the presence of the antibiotic. No calves on the same diet but without antibiotic supplement were available, but the conclusion seems to be justified, since similar differences existed between treatments 1 and 2 and treatment 3, none of which had received an antibiotic supplement, the type of diet being the only difference. Mann, Masson & Oxford (1954*b*) found that calves receiving an Aureomycin supplement had rumen contents of higher pH than control animals, but did not observe any direct effect of the antibiotic supplement on the bacterial population.

The relationship which appeared to exist between the age of the calves and the composition of the bacterial population of the rumen was most evident with the streptococci. During the first week of life the number of species was large and the typical, amylolytic streptococcus, *Strep. bovis*, amounted to only 28·6% of the total number of streptococci isolated. During the succeeding two weeks the population gradually stabilized, *Strep. bovis* becoming the predominating species, rising to over 90% of the streptococcus population after 6 weeks. This was not so in all calves: in 5 animals, irrespective of treatment, *Strep. bovis* alone was isolated. It seems, therefore, to be to some extent a matter of chance which of the streptococci present in the calves' environment develop in the rumen. In individual cases streptococci of serological groups B, E and G were also isolated, which again suggests indiscriminate colonization from the environment. However, in all calves *Strep. bovis* became the predominating species in a relatively short time, eliminating other streptococcus species. This confirms, to some extent, the observation of Mann, Masson & Oxford (1954*a*) that the ratio of cellobiose fermenting to mannitol fermenting bacteria in the rumen of calves increased with increasing age of the animals.

Among the isolated strains of *Strep. bovis* a marked variation of physiological characteristics, not normally found in strains from adult animals, was observed. Higginbottom & Wheater (1954) found remarkably little variation in morphological and biochemical characteristics among strains isolated from different animals; strains isolated by Perry *et al.* (1955) were also fairly uniform. In the present study, the variation was greater and 4 different physiological types were isolated; the incidence of these types was related to the age of the calves.

Type I seemed to predominate in older calves and adult cattle. It conformed to the descriptions given by Higginbottom & Wheater (1954) and Perry *et al.* (1955), being inconsistent with the latter's data only in respect of reaction in litmus milk. Perry *et al.* reported the production of "slight acidity"; some of the present type I strains also caused a very slight change in the colour of the litmus but, by comparison with the acid clot and reduction of the litmus produced by the three other types, these results were taken as negative. In calves, type I was found from the first day of life and predominated from the fifth week onwards.

Type II occurred only in very young calves; it predominated during the first two weeks and then decreased. Most of the strains failed to hydrolyze starch and showed slightly increased heat resistance.

Type III seemed to be typical of older calves. It occurred regularly after 8 weeks and formed 6·2 to 21·4% of the total *Strep. bovis* isolates. From the rumens of the dams only types I and III were isolated, in proportions similar to those found in the older calves. This again is an indication that the two types are typical of the adult rumen.

Type IV occurred sporadically and could not be related to the age of the calves. A similar atypical *Strep. bovis* was isolated by Mann & Oxford (1955) from the rumen of a young calf.

The differences among the 4 types did not exceed the variation normally found within the species; the most notable feature was that the variation in physiological characteristics was associated with different periods of growth of the young animal.

With other streptococcus species a marked uniformity in the results of physiological tests was evident. The *Strep. faecalis* strains resembled strains from human sources in not being raffinose fermenters; strains which were all raffinose fermenters were isolated by Mann, Masson & Oxford (1954*a*) from the sheep's rumen.

The development of lactobacilli in the rumen was less regular. During the first week of life, when the rumen flora was the most unstable, lactobacilli were not found. During the second and third weeks, *L. plantarum* was isolated most frequently, whereas from the fifth week onwards *L. acidophilus* predominated. As with the streptococci, the composition of the diet modified the total numbers of lactobacilli in the rumen but did not influence the incidence of the various species.

Some discrepancies exist between the results obtained by different workers investigating lactobacilli in the rumen of both young and adult animals. However, two facts appear well established; that the rumen supports a numerous and diverse lactobacillus population and that on a diet rich in concentrates they become the predominating organisms. No relationship has been found between the composition of the lactobacillus population and the diet or age of the animals. Mann & Oxford (1954) isolated *L. brevis*, an anaerobic variety of *L. lactis*, and several unclassifiable strains from young calves. However, in later studies the same authors (Mann & Oxford, 1955) found *L. fermenti* and, in the abomasum, *L. fermenti* and *L. acidophilus*, but none of the species isolated previously. Jensen, Smith, Edmondson & Merillan (1956), investigating 168 strains of lactobacilli isolated from the rumens of a cow and a sheep on high roughage diets, found *L. plantarum*, a variety of *L. casei* which did not ferment lactose, and *L. buchneri* to be the predominating species. They also isolated a small number of other species but considered them to be passengers. Perry & Briggs (1957), investigating the lactobacillus populations of the rumens of cows and older calves, found *L. fermenti*, *L. acidophilus*, *L. casei* and, to a lesser extent, *L. plantarum*. In the present work *L. acidophilus* was the most numerous species followed by a variety, not isolated previously, which resembled *L. brevis*, and *L. plantarum*. Only a few representatives of other species were isolated. The evidence so far obtained seems to support the view of Mann & Oxford (1955) that it is again a matter of chance which of the lactobacilli from the animals' environment becomes established in the rumen. It seems reasonable to assume that the type of diet may influence the composition of the lactobacillus population, in being both a source of infection and a substrate for subsequent development.

The data presented here suggest that the microflora of the environment influences to some extent that of the rumen, and that this influence is particularly strong during the early development of the rumen flora soon after birth of the calf. During the first few weeks of life the rumen population becomes more stabilized, the type of diet determining to a considerable extent the proportions of its various constituents.

It seems that the adult rumen remains the chief source of the rumen flora of the calf, various elements of the environment mediating in the transfer of inocula where no direct contact exists.

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A STUDY OF THE BACTERIA ON THE ROOT SURFACE AND IN THE RHIZOSPHERE SOIL OF CROP PLANTS

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SUMMARY: The numbers and kinds of bacteria on the root surface (rhizoplane) of the six crop plants, red clover, flax, oats, maize, barley and wheat, and also in the rhizosphere soil (near the roots) have been compared with the numbers and kinds in root free soil. Bacteria isolated from wheat roots have been classified in genera. All the 6 crops, especially red clover, showed a typical rhizosphere effect, and there were also more bacteria in the rhizoplane than in root free soil. Of the 6 crops, red clover had the most methylene blue reducing bacteria, and flax the highest numbers of glucose fermenting bacteria, in both rhizosphere and rhizoplane. Flax also had very many ammonifying bacteria on its roots. The percentage of strains needing amino acids for optimal growth was highest on the root surfaces, and higher in the rhizosphere than in root free soil, in every case. Wheat roots and the wheat rhizosphere yielded a higher proportion of fast growing, acid tolerant and acid producing bacteria than did root free soil. The taxonomic studies showed that *Pseudomonas* spp. were predominant on and near wheat roots, but *Arthrobacter* spp. were predominant in root free soil.

ALTHOUGH a great deal of work has been done on the microflora of the rhizosphere (Katznelson, Lochhead & Timonin, 1948; Clark, 1949), comparatively little has been reported on micro-organisms living in intimate association with normal, healthy plant roots. Yet the microflora on the root surface may be more characteristic of a plant species than that of the rhizosphere soil, in which a specific root effect may be diluted out or possibly obscured by the soil microflora. Organisms intimately associated with roots may also reflect much more precisely the changing conditions attending the growth and metabolism of a plant than would the organisms in the rhizosphere soil. The desirability of making a distinction between the rhizosphere soil organisms and those on the root surface has been suggested by a number of workers (Gräf, 1930; Poschenrieder, 1930) who referred to these zones as the outer and inner rhizosphere, and by Clark (1949) who suggested the term 'rhizoplane' to distinguish between the external surface of the plant root and the soil adhering to it. Recent reports by Sperber & Rovira (1959), Louw & Webley (1959*a*,*b*) and Vagnerova, Macura & Catska (1960*a*,*b*) further attest to the increasing interest in this zone of plant-microbe interaction.

In the studies reported below the bacterial flora of the root surface and the rhizosphere of 6 crops was compared quantitatively and qualitatively with that of root free (control) soil. The isolates from wheat roots, rhizosphere and control soil were classified in genera.

MATERIAL AND METHODS

The plants used were red clover (*Trifolium pratense*), flax (*Linum usitalissimum*), oats (*Avena sativa*), maize (*Zea mays*), barley (*Hordeum vulgare*), and wheat (*Triticum vulgare*). They were grown for 6 weeks in the greenhouse in large rectangular tanks containing uniformly mixed fertile soil to a depth of 18 in. A suspension of rhizosphere soil was obtained by shaking the roots with adhering soil in 90 ml of sterile water for 5 min. After dilutions were made the roots were carefully washed and the washings combined with the original suspension which was evaporated, and the soil was weighed. The roots were dried between filter paper and their moist weight determined. They were then shaken in three changes of sterile water, transferred to 100 ml of sterile water in a sterile Waring blendor, and a rhizoplane suspension prepared by grinding the roots for 3 min. Serial dilutions in 90 ml of sterile water were made from these two suspensions and from a suspension of root free soil. The bacteria were counted by plating on soil extract agar (Lochhead, 1940), and certain physiological groups were estimated by the dilution to extinction method in appropriate media (Katznelson, 1946; Katznelson & Rouatt, 1957). For tests of acid production 0·5% of glucose was added. All colonies from the plates used for the bacterial counts, or from suitable sectors of such plates, were picked to provide isolates for studies on nutrition, acid production and sensitivity, and growth rate (Lochhead & Chase, 1943; Rouatt & Katznelson, 1957). The isolates from the wheat series were identified according to Bergey's *Manual* (Breed, Murray & Smith, 1957) using the *Manual of Microbiological Methods* (Manual, 1957). The *Guide to the Identification of the Genera of Bacteria* (Skerman, 1959) was also used as required.

RESULTS

Quantitative. The plate counts for the 6 crops used are shown in Table 1. It may be noted that numbers of organisms in the rhizosphere and control soils are calculated on an oven-dry basis and can be compared directly. However, counts of the root surface or rhizoplane are based on both dry and wet weights, and are not directly comparable with those of the rhizosphere and control soil; the use of wet weights of roots gives lower values and the use of dry weights higher values than shown for the rhizosphere soil. Nevertheless it is clear that a typical rhizosphere effect occurs with all crops used, particularly red clover which shows a ratio of rhizosphere soil:

Table 1. *A comparison of the colony counts of bacteria in the rhizosphere and rhizoplane of different crop plants and in root free soil*

Crop	Colony count (10^6/g) of soil*		Colony count (10^6/g) of rhizoplane† based on	
	Root free	Rhizosphere	Dry wt.	Wet wt.
Red clover	134	3255	3844	320
Flax	184	1015	2450	290
Oats	184	1090	3588	290
Maize	184	614	4500	375
Barley	140	505	3216	270
Wheat	120	710	4119	340

* Based on oven dry soil. † Based on root weight.

control soil (R:S) of 24 as compared, for example, with maize with an R:S ratio of 3·3. However, rhizoplane counts provide little evidence of significant plant differences.

A comparison of certain physiological groups of bacteria, as estimated by the most probable number technique, is given in Table 2. In this Table the rhizoplane counts are given on the dry root basis; the values for moist roots may be readily determined by calculating appropriate factors with the figures given in Table 1. The numbers of methylene blue reducing bacteria were greatest in the rhizosphere and rhizoplane of red clover, whereas organisms fermenting glucose were more abundant in the vicinity of, and on, flax roots. Ammonifying bacteria were also markedly stimulated on flax roots. The rhizospheres of maize, barley and oats did not appear to stimulate acid producers markedly, but were more favourable for gas producers; in the latter case appreciable R:S ratios were obtained (for maize 15, oats 20, and barley 50). This group was markedly enhanced in the rhizosphere of red clover and flax.

Table 2. *A comparison of the viable counts of certain physiological groups of bacteria in the rhizospheres (a) and rhizoplanes (b) of different crop plants and in root free soil*

Crop		Viable counts *(10^6/g) of groups			
		Reducing methylene blue	Forming acid from glucose	Forming gas from glucose	Ammonifying
None (control soil)		40	4	0·2	30
Red clover	(a)	1900	110	60·0	340
	(b)	2800	1280	170·0	830
Flax	(a)	670	240	130·0	240
	(b)	1180	5630	1560·0	2980
Oats	(a)	390	10	4·0	100
	(b)	1190	560	95·0	310
Maize	(a)	300	5	3·0	630
	(b)	420	120	7·5	750
Barley	(a)	70	10	10·0	720
	(b)	1580	160	160·0	750
Wheat	(a)	250	200	2·5	750
	(b)	450	200	4·5	1500

* Counts of control and rhizosphere soils/g of oven dry material; counts of rhizoplane/g of oven dry roots.

Qualitative. The isolates obtained from all samples tested were grown in three media: one composed of inorganic salts and glucose (B); one composed of medium B plus Casamino acids (A); and one composed of medium B plus soil and yeast extracts (YS) (Lochhead & Chase, 1943). Very few organisms grew well in medium B, the majority finding media A and YS more favourable. The percentage incidence of those growing best in medium A is shown in Table 3. It is this group which has been consistently found to respond most favourably to the influence of plant roots. In every instance a distinct rhizosphere effect may be noted and in every case this selective effect was even more pronounced in the rhizoplane.

Previous studies in this laboratory (Rouatt & Katznelson, 1957) have shown that in the rhizosphere there is a significantly higher proportion of rapidly growing bacteria than in root free soil. As may be seen in Table 4, the wheat isolates showed the same general trend with the exception that there was no difference between the values for the rhizosphere and rhizoplane isolates. Table 4 also presents additional data which are of considerable interest. The percentage incidence of acid tolerant bacteria was greater in the root zone than in root free soil. Acid producing organisms were also more abundant in the vicinity of the roots.

Table 3. *The effect of plant roots on the incidence of bacteria requiring amino acids for optimal growth*

Crop	Incidence (%) in		
	Control soil	Rhizosphere	Rhizoplane
Red clover	10	18	31
Flax	11	54	69
Oats	11	44	55
Maize	11	14	41
Barley	10	38	64
Wheat	9	45	58

Table 4. *A comparison of isolates from the rhizosphere and rhizoplane of wheat plants with those from root free soil, with regard to growth, acid tolerance, and acid production from glucose*

Source	Isolates (%) showing		
	Good growth*	Acid tolerance†	Acid production‡
Root free soil	52	43	19
Rhizosphere	81	62	28
Rhizoplane	80	78	38

* Less than 80% light transmission after incubation for 5 days.

† Growth in YS medium as good at pH 5·5 as at pH 6·8.

‡ In a glucose medium containing bromocresol purple.

Taxonomic studies. The generic composition of the isolates from the rhizoplane and rhizosphere of wheat and from root free soil is given in Table 5. The most striking feature of this is the predominance of *Pseudomonas* spp. in the vicinity of the root and the predominance of *Arthrobacter* spp. in the control soil. As in the case of the amino acid requiring bacteria the figures for the rhizosphere soil are intermediate between those of the soil itself and of the root surface. For the remaining genera only certain trends may be mentioned, such as the tendency of *Achromobacter* spp. to increase towards the root and that of species of *Mycobacterium*, *Mycococcus* and *Mycoplana* to be more abundant away from the root. However, there were too few isolates in these genera to warrant more than a passing reference.

Lochhead (1940) has shown that Gram-negative rods and coccoid rods predominate in the vicinity of the plant root, observations which have been verified many times and most recently by Sperber & Rovira (1959) and Vagnerova *et al.* (1960*a*,*b*). The overall morphological characteristics of the wheat isolates are summarized in Table 6, and it is clear that the situation was similar in this case also, though coccoid rods were absent. The effect is most pronounced on the immediate root surface.

Table 5. *The incidence of bacteria of different genera in root free soil and in the rhizosphere and rhizoplane of wheat plants*

Genus	Incidence (%)* of genera in		
	Root free soil	Rhizo-sphere	Rhizo-plane
Achromobacter	2	4	6
Arthrobacter	45	34	22
Bacillus	7	5	5
Cellulomonas	4	2	3
Flavobacterium	2	3	3
Micrococcus	4	3	3
Mycobacterium	4	1	0
Mycococcus	5	2	0
Mycoplana	4	3	1
Nocardia	8	12	9
Pseudomonas	15	30	48

* Based on 107 cultures from root free soil, 99 from the rhizosphere and 100 from the rhizoplane.

Table 6. *The relative incidence of different morphological groups of bacteria in root free soil and in the rhizosphere and rhizoplane of wheat plants*

Gram reaction	Morphology*	Incidence (%) of morphological groups in		
		Root free soil	Rhizo-sphere	Rhizo-plane
+	Rods	9	5	5
	Coccoid rods	5	2	0
	Cocci	4	3	3
	Pleomorphic forms	42	27	13
−	Rods	25	42	61
	Coccoid rods	0	0	0
	Cocci	0	0	0
	Pleomorphic forms	15	20	18

* Based on an examination of cultures 24 hr old.

DISCUSSION

From quantitative considerations alone, the analysis of the microflora of the rhizoplane may not be particularly instructive, since no significant differences between plant species are detectable (Table 1). In this connection rhizosphere soil counts may be a better criterion of the root effect, as red clover counts were distinctly higher than those of wheat, barley and maize. This is in accord with frequent reports on the greater rhizosphere effect of leguminous plants (Katznelson *et al.* 1948). However, an examination of the qualitative data shows that a determination of the rhizoplane microflora may be a useful additional parameter for studying the influence of plant roots on soil micro-organisms. This view is supported by the material in Table 7

Table 7. *A comparison of the results of an analysis of the bacterial floras of the rhizospheres (a) and rhizoplanes (b) of six crop plants*

Physiological or nutritional group	Source	Crop plants* in order of decreasing count	Ratio of highest to lowest values
Methylene blue reducing	(a)	RC> F > O > M > W > B	27
	(b)	RC> B > F = O > W > M	7
Acid from glucose	(a)	F > W > RC> O = B > M	48
	(b)	F > RC> O > W > B > M	47
Gas from glucose	(a)	F > RC> B > O = M = W	43
	(b)	F > RC= B > O > M > W	312
Ammonifying	(a)	W > B > M > RC> F > O	7
	(b)	F > W > RC> B = M > O	99
Amino acid requiring	(a)	F > O > B = W > RC> M	4
	(b)	F > B > W > O > M > RC	2

* RC, red clover; F, flax; O, oats; M, maize; B, barley; W, wheat.

in which the data of Tables 2 and 3 are rearranged to show the crops in order of decreasing numbers of specific physiological and nutritional groups of bacteria in the rhizoplane and in rhizosphere soil. In general there appears to be good correspondence between the results of the rhizosphere soil and rhizoplane analyses. In the methylene blue series both sets of results show red clover to be the most active crop, and wheat and maize the least. Both series show that flax supported the greatest number of organisms fermenting glucose and the highest proportions of bacteria requiring amino acids for optimal growth, whereas maize and wheat were the poorest crops with regard to acid forming and gas producing organisms, respectively. The correspondence fails in only one instance, the ammonifying group, but even with this, wheat was among the most effective of the six crops in both instances. However, the rhizosphere soil showed a greater spread between the highest and lowest counts of methylene blue reducing bacteria and may be a better index for this group, whereas for gas producing and ammonifying bacteria the greatest range of numbers was obtained from the rhizoplane data. These comparisons suggest that for certain groups of organisms analysis of the rhizosphere soil may provide a more accurate picture; for other groups rhizoplane data may be more informative.

There is little doubt that for amino acid requiring bacteria (Table 3), for acid tolerant and acid producing types (Table 4) and for specific generic groups (Table 5) the rhizoplane appears to be a very favourable habitat, with rhizosphere soil occupying an intermediate position between it and root free soil. These effects are to be expected as root excretions are most concentrated at the root surface and become diluted out with distance from the root. Such a gradient of root diffusate has been recorded previously in experiments on the effect of soil desiccation on the incidence of amino acid requiring bacteria in the root zone (Katznelson *et al.* 1956). Louw & Webley (1959*a*) have also reported a higher incidence of bacteria requiring amino acids on the root surface of oats (64% of their isolates) than in the rhizosphere soil (44%). Vagnerova *et al.* (1960*a*,*b*) reported essentially similar results with wheat.

Of particular interest is the observation that the rhizoplane also supported fewer bacteria sensitive to acid and a greater proportion of acid producing organisms than root free soil, rhizosphere soil being intermediate. Lochhead (1940) recorded higher proportions of acid producers in the rhizosphere than in control soil, and Louw & Webley (1959*a*) stated that acid producers were preferentially stimulated in the root region. Most recently, Sperber & Rovira (1959) reported that the majority of the isolates from clover and ryegrass roots produced acid from glucose. These results suggest that the root zone may be acidic in nature, not only because of acidic substances which may be produced by the roots and by the rhizoplane microflora, but also because of the CO_2 produced by both roots (Meyer, Anderson & Böhning, 1960) and associated micro-organisms (Russell, 1950). The fact that most of the bacteria on the root surface were found to be acid (pH 5·5) tolerant suggests an adaptation to an acidic environment. From a practical point of view the solubilization of phosphate may also be favoured under such conditions. Vigorov (1958) suggested that one of the important factors limiting the development of *Azotobacter* spp. in the wheat rhizosphere is the unfavourable pH of the rhizosphere soil.

The generic composition of the isolates from the wheat series (Table 5) is in general agreement with the observations of Clark (1940), Vagnerova *et al.* (1960*a*,*b*) and Vozniakovskaia & Zhil'tsova (1958), but is somewhat at variance with the results of Sperber & Rovira (1959). The differences may be due simply to the nature and age of the plants used. Sperber & Rovira worked with 15-week old clover and ryegrass as compared with the seedling wheat used by Vagnerova *et al.* (1960*a*,*b*) and by the present authors. However, the Russian workers (Vozniakovskaia & Zhil'tsova, 1958) also reported a large proportion of *Pseudomonas* spp. on the roots of maize and gooseberry. Vagnerova *et al.* (1960*a*,*b*) reported this group to be the most prominent one on the root surface, with one-third as many in the control soil. Rhizosphere soil occupied an intermediate position in this regard. The proportions of *Arthrobacter* spp. were not high in their control soil (7%) whereas in the soil used in the present work this genus accounted for 45% of the isolates. The difference between these soils is further illustrated by the very high proportion of *Bacillus* spp. in the Czechoslovakian soil (36%) as compared with the Canadian soil (7%).

The abundance of Gram-negative bacteria, especially of the genus *Pseudomonas*, may be expected from a knowledge of the metabolic and growth characteristics of its representatives as isolated from the rhizoplane and from rhizosphere soil. These were found to have a shorter generation time and shorter lag period than *Arthrobacter* spp. in the same medium and produced acidic products more actively (Chan & Katznelson, 1961). The results of these workers suggest that the preponderance of pseudomonads in the root zone is due to at least three factors which may operate singly or together depending on environmental conditions: their rapid growth, their ability to produce acidic substances, and to elaborate fluorescent pigments which are decidedly inhibitory to *Arthrobacter* spp. and possibly other organisms. Quite aside from their competitive propensities, these pseudomonads are of interest because some of them at least produce 2-ketogluconate, which has been shown by Louw & Webley (1959*a*) to be important in calcium chelation and therefore possibly concerned with the solubilization of insoluble phosphate. Thus these bacteria would appear to be a most important component of the microbial population on and surrounding the roots of young, healthy plants.

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THE EFFECT OF MODIFICATIONS IN THE COUNTING MEDIUM ON THE VIABILITY AND GROWTH OF BACTERIA DAMAGED BY PHENOLS

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SUMMARY: Bacteriological agar has been shown to be toxic towards *Escherichia coli* and *Staphylococcus aureus.* The effect was observed when nutrient agar was treated with Norit or ferric chloride and when the agar component alone was treated similarly. With both agents, larger colonies from untreated suspensions of *Staph. aureus* and higher counts from phenol treated suspensions of both species, were produced. Aluminium and chromium gave effects similar to those of iron, but iron gave the largest colonies of *Staph. aureus.* Magnesium also was especially successful in inducing high counts of damaged cells.

A batch of Japanese agar, although apparently of poor quality, was superior by both the above criteria to two batches of New Zealand agar; but all three batches of agar were toxic to some degree.

In the course of investigations into the effects on the viability of cells after exposure to phenols of the inclusion of Norit or ferric chloride in nutrient media (Jacobs & Harris, 1953, 1954, 1960), it was concluded that any favourable effect that these adjuvants might have on the viability and growth of phenol treated cells was due to their action on the medium, rather than on the organisms. Thus it became of interest to see what effects these substances had when added to solid media.

Materials and Methods

The materials and methods were, generally, such of those described previously (Jacobs & Harris, 1960) as were applicable to the present work. The organisms were a laboratory strain of *Escherichia coli* type I (44°+) and *Staphylococcus aureus* NCTC 4736, and these were grown and exposed to phenol, *o*-cresol and *p*-chloro-*m*-cresol (PCMC) in the manner referred to in the earlier paper. The following points of difference are important.

Media

The nutrient broth used routinely (Oxoid peptone no. L 37, 10 g; Lab-Lemco, 10 g; sodium chloride, 5 g; tap water, 1 litre) was solidified when required by adding 15 g/litre of New Zealand agar or 25 g/litre of Japanese agar. Unless otherwise stated, the agar used routinely was the batch referred to subsequently as New Zealand 1.

Norit and ferric chloride treatments. When either or both the nutrient broth and the agar components were treated, the media were prepared as follows, all percentages being w/v.

The methods were such that the proportion of Norit and ferric chloride to medium constituents was constant throughout and, as far as possible, all media were treated identically, except for the special modifying treatment with Norit or ferric chloride.

Nutrient agar. Double strength nutrient broth was mixed with an equal volume of a double strength solution of agar in distilled water.

Norit-broth. Double strength broth was shaken with 0·2% Norit for 30 min and the Norit removed by filtration through filter paper.

Norit-agar. To 3% of New Zealand agar or 5% of Japanese agar suspended in distilled water 0·2% of Norit was added. After autoclaving the mixtures at 10 lb/in^2 for 10 min the Norit was removed by filtration through paper pulp.

Norit-nutrient agar. This was nutrient agar containing 0·1% of Norit.

Ferric chloride-broth (*$FeCl_3$-broth*). Double strength nutrient broth containing 0·06% of ferric chloride was shaken for 30 min, heated to boiling and the coagulated precipitate filtered off.

Iron-treated agar (*FeP-agar*). To 3% of New Zealand agar or 5% of Japanese agar suspended in distilled water the ferric chloride-broth precipitate (FeP) obtained from an equal volume of ferric chloride broth was added. The whole was autoclaved at 10 lb/in^2 for 10 min and filtered through paper pulp to remove the FeP. Agar cannot be treated with ferric chloride because the acidity results in hydrolysis of the agar and loss of gelling power.

Ferric chloride-nutrient agar (*$FeCl_3$-nutrient agar*). Nutrient agar containing 0·03% of ferric chloride.

To solidify Norit-broth and ferric chloride-broth, an equal volume of a double strength solution of agar in distilled water was added.

Cation treatments. In the section dealing with the improvement of nutrient agar by adding of cations, calcium, magnesium, aluminium and chromic chlorides were added to nutrient broth at the same molar concentration as 0·03% ferric chloride, i.e. 0·00185 M. The agar was then added and the medium sterilized by autoclaving. All the salts were of Analytical Reagent quality.

The assessment of the growth value of solid media

In early experiments it was obvious that colony sizes were greater with some medium treatments and it was desired to assess the magnitude of this effect, in order to see whether or not the capacity of a medium to bring about the revival of treated bacteria was related to its ability to grow them. Accordingly, a 'growth index' was calculated, this being a measure of the total area of growth on a medium, as follows.

The surface viable count was performed in the normal way with untreated bacteria and all the colonies from 5 drops of the suspension were classified into size groups in steps of 1 mm, e.g. <1 mm diameter (mean diameter assumed to be 0·5 mm), 1–2 mm (mean 1·5 mm), 2–3 mm (mean 2·5 mm), etc. The percentage colony size distribution, i.e. the percentage of the total in each size group, was then calculated and the growth index computed as the sum of the products of the percentage in a size group and the square of its mean diameter. In all cases the growth indexes were calculated from measurements made after 48 hr incubation, as differentiation was better at that time than after 24 hr.

To obtain consistent results with this technique it was necessary to control the mean count, the variation between counts, the depth of the medium and the humidity in the incubator. Determinations could not be carried out with treated bacteria since the numbers per drop varied markedly from one experiment to another and between media, and many colonies were late in developing. However, it was apparent on inspection that the growth pattern was similar from both untreated and treated suspensions. In this assessment *Staph. aureus*, which tended to form flat, spreading colonies, gave more satisfactory results than *E. coli*, because enhanced growth of the latter tended to result in more convex colonies rather than in ones with a greater diameter.

Interpretation and presentation of the data

It is well known that in disinfection experiments considerable variations occur in the results, and in this work there is an indication that the magnitude of the response in a particular experiment was greater when the control count was smaller, an effect already commented upon briefly in a previous paper (Jacobs & Harris, 1960). Because of this, and despite the occurrence of large effects, formal statistical proof of the significance of differences due to treatment was difficult to obtain. The problem of the occurrence of large variations in response between trials has been discussed by Harris, Richards & Whitefield (1961), who observed, in an examination of similar but more extensive data, that the mean counts did not follow a normal distribution. However, no greater skewness of the distribution occurred in the individual drop counts and it was concluded that no greater errors would enter into the analysis of variance if individual drop counts were used in place of the means. In the present work, if this statistical technique is followed, differences of the order of 20% are found to be significant at the 1% level. However, the authors feel that this approach may introduce bias, especially with small counts, and accordingly no statistical evidence will be presented.

The bulk of data is too extensive to permit presentation of the results of individual trials, so mean values are given in the Tables. It must be stressed that, in view of the considerable between trial variation, although the figures presented rank the media reliably in the order of their ability to grow damaged bacteria, too much reliance should not be placed on the actual magnitudes of the mean figures presented, which relate only to the particular experiment concerned.

RESULTS

The effects produced by Norit and ferric chloride in nutrient agar

In a preliminary experiment, untreated and phenol treated suspensions of *E. coli* were counted by the surface viable method on nutrient agar and on Norit-nutrient agar. There were no significant differences in the numbers of untreated cells but with treated bacteria the mean count on the Norit medium was 40 times that on the control, untreated medium.

This response to the presence of Norit seemed larger than was probable from previous experiences with liquid media (Jacobs & Harris, 1953, 1960), so it was decided to determine whether the adsorbents affected the broth, the agar or both.

To compare the effect of the adsorbents on the broth and on the medium as a whole, counts were done on nutrient agar, ferric chloride-nutrient agar, ferric chloride-broth plus agar, Norit-nutrient agar and Norit-broth plus agar. The mean results

Table 1. *The effect of ferric chloride or Norit treatments of nutrient agar and nutrient broth on the viability of untreated and bactericide treated cells and on the growth of untreated bacteria*

Organism and medium	Mean relative viable count* after treatments				Mean growth index*
	Phenol	*o*-Cresol	PCMC†	None	
E. coli					
$FeCl_3$-nutrient agar	230	462	575	101	177
$FeCl_3$-broth + agar	124	153	106	100	143
Norit-nutrient agar	220	410	400	96	203
Norit-broth + agar	117	298	226	106	172
Staph. aureus					
$FeCl_3$-nutrient agar	345	142	200	95	306
$FeCl_3$-broth + agar	117	120	105	94	238
Norit-nutrient agar	1,513	150	225	109	106
Norit-broth + agar	253	193	175	100	90

* Mean of 3 trials with one batch of media, relative to untreated nutrient agar as 100.
† PCMC, *p*-chloro-*m*-cresol.

Table 2. *The relationship between the count from suspensions of bacteria damaged by exposure to phenolic bactericides and their responsiveness to the presence of Norit or ferric chloride in the counting medium*

E. coli			*Staph. aureus*		
Control count*	Relative response† to		Control count*	Relative response† to	
	Norit	$FeCl_3$		Norit	$FeCl_3$
0·2	1,100	1,300	0·2	330	400
1·2	706	1,220	0·4	350	300
1·6	350	438	1·4	> 3,000	628
2·6	685	732	2·2	136	155
3·8	432	373	4·8	458	438
7·8	195	216	9·2	174	157
9·4	162	400	11·2	222	163
10·2	284	253	19·0	272	106
19·4	306	280	20·8	257	180
22·6	194	224	22·6	244	264
24·4	162	225	22·6	96	96
48·6	215	211	24·8	218	175

* Mean of 5 drops, on nutrient agar. † Count in the presence of 0·1% (w/v) of Norit or 0·03% (w/v) of ferric chloride as percentage of control count.

of 3 trials with one batch of medium, which confirmed the previous findings, are given in Table 1. A single trial with another batch of medium gave similar results. When present in the medium both Norit and ferric chloride gave uniformly increased counts of treated organisms, but when the nutrient broth had been treated and the adsorbent removed the effect was variable. With untreated suspensions, though the counts were substantially the same on all the media, there was a beneficial effect on the growth index for *Staph. aureus* with both the iron treated media and possibly also when Norit was present in the medium: when only the broth had been treated with Norit the results were irregular. Ferric chloride in the medium gave better results than treatment of broth alone followed by its removal, and both iron treatments were consistently superior to those with Norit in inducing larger colonies. With *E. coli*, although the overall responses seem to have been favourable, the individual growth indexes varied a good deal. This was also the case with Norit and *Staph. aureus*.

Table 2 gives some details of the results summarized in Table 1 and illustrates not only the large variations in response between trials but also that large responses tended to be associated with low control counts, although this association is very imperfect.

Table 3. *The effect of separate treatment with Norit or iron of the nutrient broth and agar components of a solid medium on the viability of untreated and bactericide treated bacteria, and on the growth of untreated bacteria*

Organism and medium	Mean relative viable count* after treatments				Growth index*‡
	Phenol†	*o*-Cresol†	PCMC†	None‡	
E. coli					
Nutrient broth + Norit-agar	148	151	239	95	109
Norit-broth + Norit-agar	239	198	259	94	74
Nutrient broth + FeP-agar	218	195	235	98	114
FeCl$_3$-broth + FeP-agar	343	227	368	105	66
Staph. aureus					
Nutrient broth + Norit-agar	216	209	350	105	102
Norit-broth + Norit-agar	340	214	334	98	102
Nutrient broth + FeP-agar	264	225	624	114	257
FeCl$_3$-broth + FeP-agar	272	277	654	107	272

* Relative to nutrient agar control as 100. † Mean of 2 trials with one batch of medium. ‡ Results of a single trial.

Further counts were then done on nutrient agar, nutrient broth plus Norit-agar, Norit-broth plus Norit-agar, nutrient broth plus iron-treated agar and ferric chloride-broth plus iron-treated agar. The results, given in Table 3, confirm that treatment of the agar alone with Norit or FeP gave higher counts than the control, and that in general the use of a treated broth with the corresponding treated agar tended to increase the counts over those obtained when only the agar had been treated. Once again, the counts from untreated suspensions were similar on all the media. Only *Staph. aureus* gave enhanced growth indexes and these were confined to the iron-treated media.

A comparison of New Zealand and Japanese agars, and their improvement by adsorbents

At this stage it became clear that a considerable proportion of the inhibitory effect of nutrient agar was attributable to the agar component, and it was decided to find out what variation might be expected between agars from different sources and of different batches and how far it might be possible to improve them by treatment with Norit or FeP.

Three batches of agar were chosen, one of Japanese, used at 25 g/l, and two of New Zealand (NZ1, NZ2), used at 15 g/l. Double strength sols of these agars in water were divided into three parts, one of which was treated with Norit and another with FeP. All three portions were autoclaved, filtered through paper pulp and diluted with an equal volume of the identical double strength nutrient broth. Plates were prepared from each of these nine media for use in counting by the surface viable method. Both organisms, untreated and treated with all three phenols, were counted on all nine media, and the mean results of three trials with each bactericide and those of a single trial with untreated bacteria are given in Table 4. From this it can be seen that treatment of any of the agars with either adsorbent almost always improved the counts of treated *Staph. aureus*: but, whereas with *E. coli* iron treatment of all the agars gave higher counts from treated suspensions, Norit improved the first

Table 4. *The effect of different lots of agar, and treatment of them with Norit or FeP, on the viability of untreated and bactericide treated bacteria on solid media prepared from these agars, and on the growth of untreated bacteria*

Organism, type of agar and agar treatment		Mean relative viable count* after treatments				Mean growth index‡
		Phenol†	*o*-Cresol†	PCMC†	None‡	
E. coli						
Japanese	None	100	100	100	100	100
	Norit	47	75	33	105	87
	FeP	276	227	777	100	84
NZ 1§	None	1	23	5	99	113
	Norit	109	168	105	105	171
	FeP	25	93	53	92	107
NZ 2§	None	1	11	3	92	137
	Norit	1	8	1	108	140
	FeP	118	169	39	106	119
Staph. aureus						
Japanese	None	100	100	100	100	100
	Norit	385	457	185	107	89
	FeP	347	497	244	102	98
NZ 1§	None	91	93	83	109	31
	Norit	145	123	101	105	43
	FeP	94	141	107	110	72
NZ 2§	None	29	24	32	99	44
	Norit	53	54	47	114	39
	FeP	285	370	97	98	70

* Relative to the medium prepared with untreated Japanese agar as 100. † Mean of 3 trials with one batch of medium. ‡ Results of a single trial. § New Zealand agars, batches 1 and 2.

batch of New Zealand agar only and appeared to impair the performance of the other agars. In the case of untreated *Staph. aureus*, iron treatment of the New Zealand agars gave the most marked improvement in growth, but neither agent improved the growth capacity for that organism of a medium prepared with Japanese agar which, without treatment, gave large colonies.

The Japanese agar, although of poorer gelling power, was superior to the New Zealand agars both in respect of the viability of treated suspensions and of the growth of *Staph. aureus* colonies on broth solidified with it. However, all three lots of agar appeared toxic to some degree since treatment with one or both adsorbents improved the ability of media containing them to grow treated bacteria.

Plate 1 illustrates the differences in colony size and in the counts of bactericide treated cells which are described above.

The improvement of nutrient agar by cations other than iron

To determine whether the effect of iron was specific, experiments were done with other cations (all as chlorides) at the same molar concentration as 0·03% (w/v) ferric chloride, namely 0·00185 M. Following promising results in preliminary trials, calcium, magnesium, aluminium and chromium were chosen for further investigation. and surface viable counts were done on media containing those ions. The grand mean results of these experiments are given in Table 5, together with some results with iron for reference. Clearly, none of the ions had any appreciable influence on the viable counts of untreated bacteria; but, whereas calcium in the concentration used caused some restriction of the growth of *Staph. aureus*, all the other ions gave higher growth indexes with that organism, iron being the best. With *E. coli*, all the ions seemed to lower the growth index.

Table 5. *The effect of adding various salts to nutrient agar on the viability of untreated and bactericide treated cells, and on the growth of untreated bacteria*

Organism and added cation*	Mean relative viable count† after treatments				Mean growth index†‡
	Phenol‡	*o*-Cresol‡	PCMC‡	None‡	
E. coli					
Calcium	462	326	100	108	83
Magnesium	17,600	17,000	4,950	89	85
Aluminium	1,580	2,260	188	106	82
Chromic	4,050	2,860	517	101	69
Ferric	3,340	1,760	717	92	86
Staph. aureus					
Calcium	365	157	90	95	11
Magnesium	1,610	620	312	98	134
Aluminium	378	272	177	101	106
Chromic	273	265	148	94	121
Ferric	476	342	147	102	264

* All cations as chlorides, at 0·00185 M. † Relative to nutrient agar without added salt, as 100. ‡ Means of 4 trials with phenol, 3 with *o*-cresol and 2 each with PCMC and untreated cells.

With treated bacteria, all the cations except calcium consistently gave increased counts of both species, magnesium being the most active, and even calcium was more often favourable than not.

No evidence was obtained that phosphate removal was responsible for these effects.

Discussion

The fact that treatment of the agar component of nutrient agar with Norit or ferric chloride-broth precipitate resulted in increased counts of bactericide treated bacteria, and larger colonies, is evidence of the presence in agar of substances toxic especially to damaged cells, such as have already been demonstrated in broth constituents (Jacobs & Harris, 1960). Further evidence that broth constituents are toxic is also afforded by the present work, since a medium prepared from treated broth and treated agar, or which contained Norit or ferric chloride, gave higher counts than one in which only the agar component had been treated.

There are few references in the literature to the occurrence of antimicrobial toxicity in agar. The inhibition of *Clostridium histolyticum* by concentrations of agar greater than 0·1% was reported by Hitchens (1921), who attributed the effect to its sulphate content. The effect of agar concentration is mentioned also in the Report (1956), which refers to the complete inhibition of some species of bacteria by 3% of an agar of which 2% permitted normal growth. Stehle (1932) observed that agar inhibited the growth stimulation of *Aspergillus niger* by zinc sulphate, while Itano & Tsuji (1934) found that some lower grade agar was more favourable to the growth of *Azotobacter chroococcum*, *Bacillus subtilis* and *Saccharomyces cerevisiae* than was agar of higher quality.

Evidence concerning the nature of the toxic agents in agar is contradictory. Ley & Muellar (1946) observed the presence of inhibitors for *Neisseria gonorrhoea* and were able to extract the inhibitory material with methanol or neutralize it by adding starch. Also, solvent extracts of seaweed have been shown to inhibit some bacterial species (Saito & Sameshima, 1955). However, Lynn & Morton (1956) found that the inhibition of some pleuropneumonia-like organisms by some batches of agar was little affected by solvent extraction of the agar or the inclusion of adsorbents in the medium. Ley & Meullar (1946) tentatively suggested that their inhibitors were fatty acids, and these acids have been implicated as toxic agents in media by other workers also (Foster & Wynne, 1948; Murrell, Olsen & Scott, 1950; Olsen & Scott, 1950; Hardwick, Guirard & Foster, 1951), who have shown in addition that they could be inactivated by the use of starch. In the present work starch did not influence the inhibitory effects, so fatty acids do not appear to be implicated. Proom, Woiwod, Barnes & Orbell (1950) associated an inhibition of *Shigella dysenteriae* which they observed on agar media with the presence of peroxides, formed when the agar and broth were autoclaved together. Taylor (1957) also observed a similar inhibition of *Bacillus* spp. on the surface of agar media, and in both these cases the inhibition could be reversed by manganese dioxide and catalase. However, in subsequent experiments on the possible implication of fatty acids (Harris & Richards, to be published) and peroxides (Harris & Whitefield, to be published) neither agent has appeared to be implicated in the type of effect reported in the present paper.

The antagonization of the toxicity by cations suggested that the observed effects may have been due to the presence of heavy metals in the medium, especially in the agar, and semi-quantitative spectrographic analyses of the ashes of the New Zealand and Japanese agars have revealed the presence of heavy metals. The Japanese agar contained more manganese and chromium than the New Zealand agar but less titanium, iron, vanadium, zinc, lead and tin, and although these differences may account in part for the lower toxicity of the Japanese material, the indications are slight.

The occurrence of medium toxicity and possible mechanisms for its alleviation have already been discussed by Jacobs & Harris (1960).

The authors wish to thank Dr. A. P. Millman for carrying out the spectrographic analyses and Dr. M. E. Solari for advice on the statistical analyses of the results.

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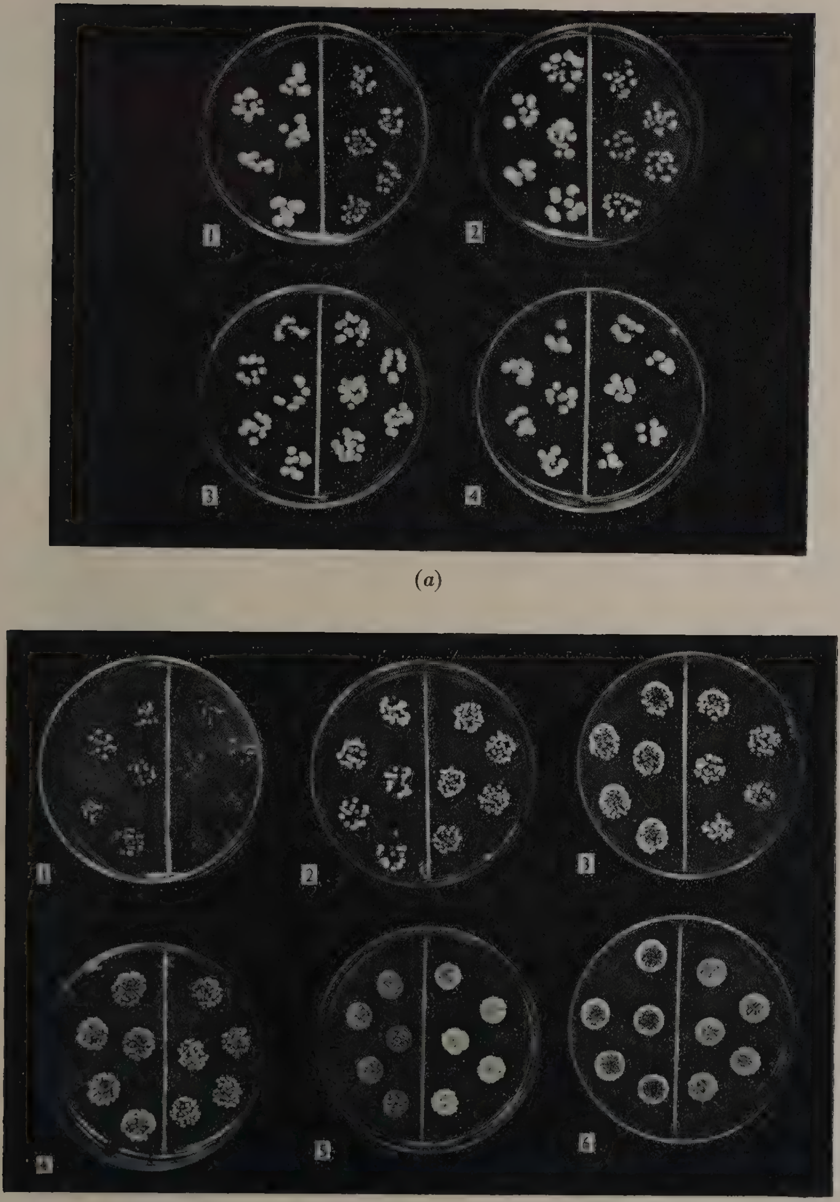

(*a*)

(*b*)

S. E. Jacobs & N. D. Harris — The Effect of Modifications in the Counting Medium on the Viability and Growth of Bacteria Damaged by Phenols. Plate 1

EXPLANATION OF PLATE

The photographs illustrate the effects obtained when suspensions of untreated or *o*-cresol treated bacteria were counted by the surface viable method on broth solidified with different agars, or with agars which had been treated with Norit or iron (FeP).

Section (a) shows the increase in colony size obtained with untreated bacteria, particularly with *Staph. aureus*, when Japanese agar was used or when the agars had been treated with Norit or FeP. Section (b) illustrates primarily the increase in colony numbers which occurred when *o*-cresol treated bacteria were counted under similar conditions, but the effect on colony size is also apparent here.

In each plate the left half received 5 drops of a diluted suspension of *E. coli* and the right half 5 drops of *Staph. aureus*, the same suspension being applied to all the plates relevant to the experiment.

The agars used, and their treatments, were: 1–3, New Zealand agar, batch 1 (NZ1); (1) untreated, (2) treated with Norit, (3) treated with iron (FeP): 4–6, Japanese agar; (4) untreated, (5) treated with Norit, (6) treated with iron (FeP).

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ON THE RELATIONSHIP BETWEEN THE VIABILITY OF BACTERIA DAMAGED BY ANTISEPTICS AND THEIR RESPONSIVENESS TO MODIFICATIONS OF THE COUNTING MEDIUM

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SUMMARY: Counts of phenol treated *Escherichia coli* and of *Pseudomonas aeruginosa* exposed to phenylmercuric nitrate varied with the medium, and the differences were greater when the mortality of the suspension was higher. The regression of the logarithm of the response (the count on a modified medium divided by the count on the control) on the logarithm of the viable count on the control was linear. This relationship is shown to occur when the log survivor-time curves from one suspension under two different conditions are divergent. To compare responses from different trials, dose reduction factors may be used or a correction may be made for the mortality level. Alternatively the mortality level should be controlled.

During investigations into some of the factors affecting the viability of damaged bacteria, in which cells treated with bactericide were counted on several media, Jacobs & Harris (1960, 1961) observed that there was a tendency for large responses to occur when counts from treated suspensions on the control medium were low, although the correlation was very imperfect.

Such an increase in response with mortality has also been observed by other workers who have used cells damaged by X-rays as well as by chemical agents. Roepke & Mercer (1947) counted X-irradiated *E. coli* on three different media and found that all gave similar counts at low mortalities, but significantly different counts at high mortalities; Alper & Gillies (1958) have reported similar results. Stapleton (1955) incubated *E. coli* at different temperatures following X-ray exposure and found that 18° was optimal for recovery. Inspection of these data shows that the greater the dose, and therefore the mortality, the greater the relative beneficial effect of incubation at 18°. Remarkable differences between the counts on two different plating media of *E. coli* B/r treated with chlorine were observed by Milbauer & Grossowicz (1959), who found that the reactivation ratio increased with the time of exposure to the hypochlorite solution. Similarly, Nash & Hirch (1954) showed that the proportion of a *Staphylococcus albus* culture which survived formaldehyde treatment in the presence of an inactivator (dimedone-morpholine) increased with increasing mortality.

Thus it appeared that the effect reported by Jacobs & Harris (1960, 1961) may have been a real one with general significance and, in the course of further studies in the same field, it was of interest to test this relationship.

MATERIALS AND METHODS

The techniques used have been described previously by Harris & Richards (1958) and by Jacobs & Harris (1960, 1961). The organisms used were the two strains (A and B) of *Escherichia coli* employed by Harris & Richards (1958), and referred to in the present paper as strains I and II, respectively, and a laboratory strain of *Pseudomonas aeruginosa* which gave small, smooth colonies. Nephelometrically standardized suspensions from a 24 hr growth on agar were treated with bactericide and counted by the surface viable method on a medium prepared from Lab-Lemco broth granules (Oxoid, CM 15), at a concentration of 16 g/l., and solidified with a suitable agar.

Four experimental situations were examined.

System 1. *E. coli* strain I was exposed to 1% (w/v) phenol and counted on the nutrient broth solidified with 15 g/l. of New Zealand agar (control) and on the same medium containing 0·00185 M ferric chloride.

System 2. The conditions were similar to those in system 1 except that the test medium contained 0·00185 M chromic chloride in place of ferric chloride.

System 3. *Ps. aeruginosa* was exposed to 0·001% (w/v) phenylmercuric nitrate (PMN) and counted on the nutrient broth solidified with 20 g/l. of Japanese agar (control) or with 15 g/l. of New Zealand agar.

System 4. The conditions of test were similar to those in system 3 except that the test medium consisted of the nutrient broth solidified with Japanese agar and containing 0·00185 M ferric chloride.

The mortalities with phenol treatments exceeded 90% and those with PMN exceeded 50%. These four systems were used because sufficiently extensive data for them were available to enable the relationships to be tested satisfactorily.

RESULTS AND DISCUSSION

System 1. In each of the 14 trials carried out, counts of phenol treated *E. coli* strain I were invariably higher when ferric chloride was present in the medium and an analysis of variance of the results showed that this difference was significant (variance ratio 894·6, for 1 and 229 degrees of freedom; $P<0·001$). Also the significance of the medium-trial interaction indicates that the response varied significantly between trials (variance ratio 260·3, for 12 and 229 degrees of freedom; $P<0·001$). This confirmed the experience of Jacobs & Harris (1960, 1961), and inspection of the data confirmed their observation that the differences in the counts between the control and test medium were related inversely to the count on the control. When the logarithm of the response (the proportional count on the test medium) was plotted against the logarithm of the count on the control medium, it was found that there was a linear relationship (Fig. 1a). Since the suspension had been standardized nephelometrically, the control count was a measure of mortality and it must be concluded that the degree of mortality of the suspension influenced the observed differences between the media. Although there is considerable scatter of the points about the line in Fig. 1a, the correlation coefficient (r) proved to be 0·92 ($P<0·001$), indicating that the straight line is a good representation of the data.

It was apparent that such a relationship could hardly be fortuitous and that a similar situation might be expected to occur in other circumstances where differential counts are obtained from a single suspension. Inspection of the data from three other experimental situations, which were under investigation at the time, revealed similar relationships.

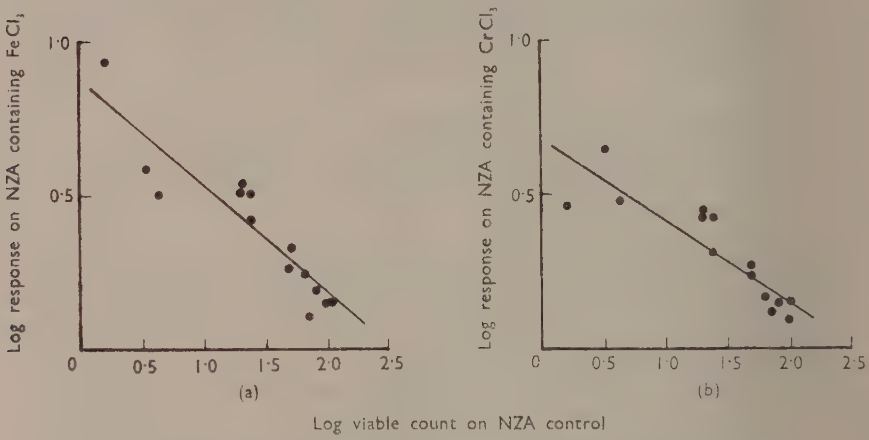

Fig. 1. The relationship of the logarithm of the response to the logarithm of the control count with *E. coli* strain I treated with phenol: organisms recovered on broth solidified with New Zealand agar (NZA) with and without FeCl$_3$ (a), and with and without CrCl$_3$ (b).

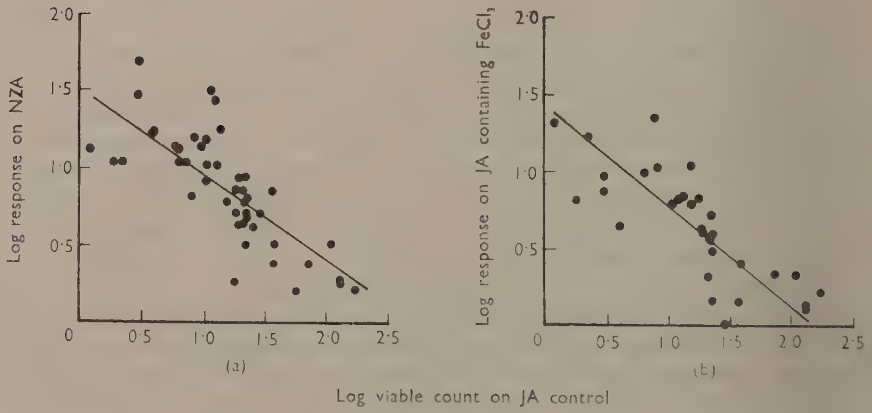

Fig. 2. The relationship of the logarithm of the response to the logarithm of the control count with *Ps. aeruginosa* treated with phenyl mercuric nitrate: organisms recovered on broth solidified with Japanese agar (JA) or New Zealand agar (NZA) (a), and on JA with and without FeCl$_3$ (b).

Other systems. With system 2, counts of phenol treated *E. coli* strain I were invariably higher in the presence of the chromic salt, and the results are summarized in Fig. 1b. Once again the trend of the results was well represented by a straight line (r, 0·89; $P<0{\cdot}001$).

Figs. 2a and 2b show the results obtained with PMN treated *Ps. aeruginosa*. Higher counts were obtained on New Zealand than on Japanese agar (system 3) and when ferric chloride was added to the medium (system 4). Here also straight lines fitted the points well, giving correlation coefficients of 0·77 ($P<0{\cdot}001$) for system 3 and 0·80 ($P<0{\cdot}001$) for system 4.

It is accepted that, in many cases, a rectilinear log survivor-time curve is a good representation of data obtained in disinfection experiments involving chemical agents, heat and ionizing radiations, although deviations from linearity also occur. If a linear relationship of this sort holds good, two straight lines diverging from a common point of origin on the ordinate axis may be obtained when samples from a bactericidal reaction are counted under two sets of conditions which yield different counts. Assuming that this relationship occurs, let us consider two media, one (medium 1) giving higher counts than the other (medium 2).

The equation for the line which represents the disinfection process as shown by medium 1 would be :

$$\log x_1 + at - b = 0$$

where x_1 is the number of survivors, t the time, a the regression coefficient and b the log of the initial count (a constant). Similarly, for medium 2 the equation would be:

$$\log x_2 + ct - b = 0$$

where x_2 is the number of survivors and c the regression coefficient. Subtracting the second equation from the first gives a third equation

$$\log \frac{x_1}{x_2} = -at + ct$$

which, on substituting the value of t obtained from the second equation, becomes

$$\log \frac{x_1}{x_2} = -\left[\frac{c-a}{c}\right]\log x_2 + \frac{b(c-a)}{c}.$$

The final equation is that of a straight line relating the logarithm of $\frac{x_1}{x_2}$ (the response) to the logarithm of x_2 (the count on the control medium), with regression coefficient $\frac{a-c}{c}$; the intercept on the abscissa is b and that on the ordinate $\frac{b(c-a)}{c}$.

Thus it may be deduced that the experimental relationships illustrated in Figs. 1 and 2 result from a situation where the log survivor-time curves for the two counting conditions are divergent straight lines.

To test this experimentally, *E. coli* strain II was exposed to 0·8% (w/v) phenol and the log survivor-time curves established for several media. *E. coli* strain I could not be used because suspensions of it had become unstable. The results of one experiment, in which counts were done on nutrient broth solidified with New Zealand agar, on the same solid medium containing 0·001 M ferric chloride and on the broth solidified with Japanese agar are shown in Fig. 3. Although all the media gave similar counts in the early stages of the reaction, three divergent linear regression lines (which should have occurred if the theory were correct) were obtained.

It is realized that disinfection experiments frequently result in curvilinear log survivor-time curves (e.g. Olsen & Scott, 1950; Alper & Gillies, 1958, 1960), and that in such circumstances the relationship deduced above cannot be expected to hold. The results of Olsen & Scott (1950) with heat treated *Clostridium botulinum* spores counted on two different media yielded semi-logarithmic regression lines which are concave downwards and diverge gradually, and indicate that the response increased with increasing mortality. On the other hand, Alper & Gillies (1958, 1960) showed that the log survivor-time curves of *E. coli* B irradiated with ultraviolet light and recovered under various conditions were sometimes concave upwards, and tended to become parallel. Here the response tended to increase with mortality in the early stages of the reaction, but it then decreased.

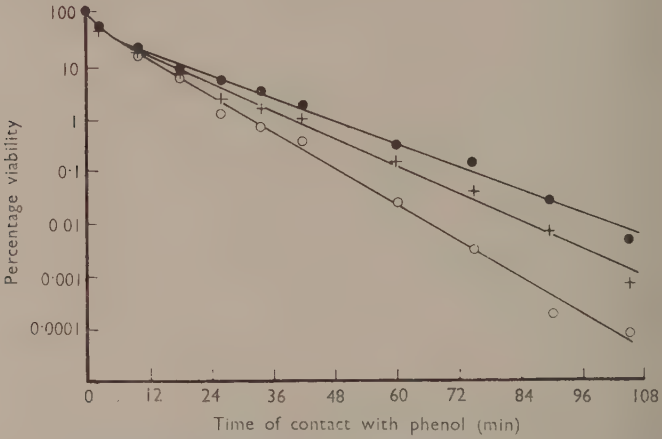

Fig. 3. Log survivor-time curves for *E. coli* strain II recovered on broth solidified with New Zealand agar and containing 0·001 M $FeCl_3$ (full circles), Japanese agar (crosses), and New Zealand agar (open circles).

Thus, where log survivor-time curves deviate considerably from linearity the way in which the response is related to the mortality will depend markedly on the shape of the curves, but where the curves are divergent the response must be expected to increase as the mortality increases.

The problems of presenting and handling the data remain, because large variations in the response make conventional statistical treatments inapplicable (see Jacobs & Harris, 1961) and make the averaging of results of several trials dubious. Three procedures are available: the use of dose reduction factors, correction for mortality level, and control of mortality level.

The dose reduction factor (DRF) is the ratio of the doses required to give the same mortality level under the different conditions; this is a constant and is independent of the mortality level. Its value may be calculated from the slopes of the log survivor-time curves or from the regression coefficient of the log response-log control count line (i.e. regression lines of the type plotted in Figs. 1 and 2) since, using the notation of the previous equations,

$$\mathrm{DRF} = \frac{c}{a} = 1 \Big/ 1 - \left(\frac{c-a}{c}\right).$$

The DRFs for the data illustrated in Figs. 1a, 1b, 2a and 2b are 1·56, 1·35, 2·33 and 2·94, respectively. Thus, where the log survivor-time curves are well represented by straight lines, the DRF may readily be calculated. It is necessary, however, to obtain more information than is given by counts at arbitrary, convenient mortalities under various conditions. If the parameters of the regression lines are known, the second approach may also be used and the response can be corrected for mortality by calculating the values at any convenient mortality level. However, if the curves are not rectilinear, neither of the above techniques is applicable, or only doubtfully so. In addition it may be impracticable to establish the exact course of the reaction. In such cases the third approach, namely the control of mortality, is highly desirable if results are to be compared.

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TWO TYPES OF XYLAN FERMENTING BACTERIA FROM THE SHEEP RUMEN

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SUMMARY: Different types of media containing xylan were used to culture bacteria from the sheep rumen. The results suggest that facultatively anaerobic bacteria are not active in the ruminal fermentation of xylans. Gram-negative curved rods were the predominant bacteria growing in all anaerobic cultures under CO_2 and those isolated were similar to *Butyrivibrio fibrisolvens*. The properties of two types of xylan fermenting bacteria are given.

Xylans can constitute a large part of the hemicellulose fraction of grass and a nutritionally important amount of xylan is fermented in the rumens of sheep feeding on grass or hay (Heald, 1953). Howard (1957) showed that washed suspensions of mixed rumen bacteria would hydrolyze xylans to oligosaccharides and finally to xylose, the latter being fermented by the mixed bacteria to volatile fatty acids (Pazur, Shuey & Georgi, 1958). Many rumen bacteria will ferment xylose, and a number of bovine rumen bacteria have been shown to decompose xylans during fermentation tests, although they were isolated in media containing other carbohydrates. The isolation of bacteria by the use of media containing only xylan as carbohydrate source, with the purpose of selecting the most active xylan fermenting bacteria, has been attempted only in some experiments on bovine rumen bacteria (Butterworth, Bell & Garvock, 1960). The enzymes concerned in, and the products of, the breakdown of xylans by a bovine rumen bacterium (*Butyrivibrio* sp.) (Butterworth, Bell & Garvock, 1960) and a sheep rumen bacterium (*Bacteroides amylogenes*) isolated in a medium containing xylose (Doetsch, Howard, Mann & Oxford, 1957), have been studied by Howard, Jones & Purdom (1960). The present paper describes a continuation of these studies directed towards the isolation of xylan fermenting sheep rumen bacteria. A number of media were tried to see if it were possible to isolate a variety of bacteria, including facultative and less strict anaerobes as well as the more specialized types of rumen bacteria needing anaerobiosis under CO_2 and often rumen fluid in the medium.

Methods

Materials. Reagents were the usual A.R. or laboratory chemical grades. Two pentosans were used. One was a wheat flour pentosan containing xylose and arabinose units. This is designated as 'xylan' throughout the paper. The other, called 'Rhodymenan', was the soluble xylan of the seaweed *Rhodymenia palmata*. Both pentosans were used by Howard, Jones & Purdom (1960). Carbohydrates were added to the autoclaved media as filter sterilized concentrated solutions, except for the pentosans which were sterilized by steaming and the starch which was autoclaved. Rumen fluid for medium 1 was prepared by straining off the coarser debris only. For medium 3 a clear fluid was obtained by centrifuging at 62,000 *g*. The fluid was kept for not more than a few days at 2° before use.

Media. Medium 1 was the solid, 10% (v/v) rumen fluid. 0·25% (w/v) xylan medium used by Butterworth, Bell & Garvock (1960). Tenfold dilutions of rumen samples were made in an anaerobic diluting fluid as described by these authors and inoculated into tubes of the medium.

Medium 2 was a semisolid one without rumen fluid like that used by Hobson, Mann & Oxford (1958) but with the sodium lactate replaced by 0·5% (w/v) of xylan. Tenfold dilutions of rumen contents were made directly in the medium.

Medium 3 was based on that of Bryant & Burkey (1953) modified to contain 40% (v/v) of clear rumen fluid, 0·4% (w/v) of $NaHCO_3$ instead of Na_2CO_3, and 0·5% (w/v) of xylan in place of glucose and cellobiose. The pH of the medium was 6·8. Tenfold dilutions of rumen contents were made in the anaerobic fluid described by these authors and inoculated into the medium.

General methods. Medium 2 was incubated in air in test tubes fitted with cotton wool plugs. Media 1 and 3 were thoroughly gassed with oxygen-free CO_2 during preparation and were dispensed and incubated under CO_2 using techniques based on those of Hungate (1950). Small amounts (10 ml) of these media were incubated in test tubes and larger amounts in bottles, both types of container being stoppered with rubber bungs. Incubation was always at 38°. Biochemical tests of the anaerobic bacteria were made in media of the same composition as media 1 and 3 but without agar and with the appropriate substrate added. Tests for motility of isolates were done on wet preparations of cultures grown overnight in liquid medium 3 containing 0·1% (w/v) of glucose, which were examined by darkground illumination.

Analysis of culture media. The degree of utilization of xylose or xylan was determined by the methods described by Howard, Jones & Purdom (1960). Volatile fatty acids were determined quantitatively by steam distillation and chromatography on Celite (Johns Manville, London, S.W.1) columns (Bueding & Yale, 1951), and qualitatively by paper chromatography of neutralized steam distillates (Elsden & Lewis, 1953). Formic acid was determined by the method of Grant (1947), lactic acid by the method of Barker & Summerson (1941), succinic acid by a manometric method (Umbreit, Burris & Stauffer, 1957) and ethanol by the method of Conway (1957).

Sampling. Samples were taken via a rumen cannula from two sheep on a daily diet of 900 g of hay and 450 g of grass cubes fed in two lots, at 7 a.m. and 4 p.m. Samples were generally taken at 11 a.m. and each sample was lightly strained to remove the coarser particles before dilutions were made.

RESULTS

Samples were taken from the two sheep over a period of some months. Observation of the types of organisms growing in each medium were made in cases where no isolations were made. The two sheep gave similar results and those described below are typical of each medium.

The bacteria growing in medium 1 were mainly Gram-negative curved rods, as in the experiments described by Butterworth, Bell & Garvock (1960). These were tested for xylan fermentation by transferring them to a similar liquid medium and purified by dilution into the solid medium. A number of xylan fermenting isolates were obtained from cultures inoculated with 10^{-4}–10^{-8} ml volumes of rumen contents. These appeared similar to the Gram-negative rods isolated from the bovine rumen (Butterworth, Bell & Garvock, 1960) in morphology, growth characteristics and fermentation of xylose and xylan, and so were not studied in detail.

Medium 2 favoured the growth of facultatively anaerobic bacteria, these being mainly cocci similar in morphology to *Streptococcus bovis*, and Gram-positive rods. Isolations of these were made from cultures inoculated with 10^{-6}–10^{-9} ml volumes of rumen fluid, but the pure cultures were found to ferment xylan only weakly, or not at all, and so were not studied further.

The initial dilutions of rumen fluid were cultured in roll tubes of medium 3. Colonies were transferred from tubes inoculated with 10^{-6}, 10^{-7} and 10^{-8} ml quantities of rumen fluid into the same medium without agar. Xylan was fermented by these cultures, but the growth was mixed, consisting of Gram-negative curved rods with some Gram-negative oval cocci and rods, Further dilutions from these cultures into roll tubes and then into liquid media (to test for xylan fermentation) were made, and from the xylan fermenting cultures finally obtained, two representative cultures from the original 10^{-6} and 10^{-7} dilutions of rumen contents were selected for detailed examination. Their properties are described below.

Both organisms were strict anaerobes and on first isolation utilized over 95% of the xylan in liquid medium 3 after incubation for 5 days. After a number of subcultures on the medium containing xylan one culture, a coccobacillus, appeared to lose its ability to grow on, and utilize, xylan, only some 25% being used. However, after rapid transfer through liquid media containing glucose it regained its ability to grow rapidly and ferment xylan. The two types were as follows.

The first was a Gram-negative curved rod which exhibited uniformity in size and staining in any one medium, but varied slightly in different media. The cells were 1·7–3·0 $\mu \times 0{\cdot}5$ μ in films stained by Gram's method. Growth in liquid cultures was granular and tended to sediment out; on slopes the growth was whitish and was best where the inoculum had been stabbed into the butt of the slope. In agar, colonies were small and lenticular. Stock cultures were kept on xylan slopes at 2° and subcultured every 2 months, or at −60°, when they survived for many months.

Nitrate reduction, Voges-Proskauer reaction, hydrogen sulphide and ammonia production and urea and gelatin hydrolysis were all negative. The indole and methyl red tests were positive. No motility and no iodophilic intracellular polysaccharide or extracellular slime were observed. The bacteria would grow in a medium in which rumen fluid was replaced by 1% (w/v) of yeast extract and this medium was used to determine the products of fermentation of xylose. Substrates fermented were: (1), xylose, arabinose, glucose, fructose, mannose, sucrose, lactose, maltose, melibiose (limited only), cellobiose, raffinose, dextrin, glycerol (limited only), glycogen, starch, xylan, mannitol, glucuronolactone and glucosamine hydrochloride; (2), galactose, trehalose, inulin, salicin (limited only), aesculin and rhodymenan. Substrates which were not fermented were: (3), rhamnose, sorbose, sorbitol, dulcitol, inositol, adonitol, lactate, succinate, fumarate, citrate, malate, and α-methyl-D-glucoside. The pH values of media in which substrates were fermented fell to 5·3, whereas when substrates were fermented to a limited extent only the pH values did not fall below 5·8–5·9.

In one experiment the products of fermentation of xylose (expressed as μ moles/ml of medium) were: acetic acid, 18·9; butyric acid, 5·9; formic acid, 5·2; lactic acid, 15·7. These products accounted for only 78·3% of the xylose carbon utilized. Propionic or succinic acids or ethanol were not formed. The organism was a vigorous gas producer and this may account for some of the unrecovered carbon. The gases produced were not examined. The volatile fatty acids produced from glucose in a 10% (v/v) rumen fluid medium were examined qualitatively and shown to be mainly butyric, with some acetic and a little propionic. The organism could thus be classified as a species of *Butyrivibrio*.

The second organism was a Gram-variable coccobacillus. The Gram-negative forms were from 0·4–2·5 μ×0·5 μ in size, generally in the more oval form. Some Gram-positive forms were also seen and these tended to be more rodlike. Forms intermediate between these were seen and dilution of cultures did not separate the two forms, the numbers of which appeared to fluctuate randomly. Growth in liquid culture was evenly turbid, and in agar roll tubes the colonies were small and surrounded by an iridescent area. Stock cultures were kept in the same way as those for the organism described above.

Nitrate reduction was variable. The indole and methyl red tests were positive and the Voges-Proskauer reaction, hydrogen sulphide and ammonia production and urea hydrolysis were all negative. Gelatin was liquefied. Motility was not observed. The bacteria would grow in a medium without rumen fluid and the products of fermentation of xylose were determined in the same medium as that used for the other organism studied.

Substrates of group (1) above were fermented, and those of groups (2) and (3) were not fermented. The pH values of the media in which substrates were fermented fell to about 4·6.

In one experiment the products of fermentation of xylose (expressed as μ moles/ml of medium) were: acetic acid, 49·6; propionic acid, 11·5; butyric acid, 1·1; formic acid, 9·1; succinic acid, 8·1. Lactic acid was not formed. These products accounted for 101·9% of the xylose carbon fermented. The products of fermentation of glucose (in 10% rumen fluid) were mainly acetic and propionic acids with little butyric. The organism was not classified.

DISCUSSION

Although bacteria not fermenting xylan will grow on the basal media or on the products of breakdown of xylan by other bacteria, the media used here should have favoured the growth of bacteria able to utilize xylan. As well as the media described above, a rich anaerobic medium (without rumen fluid) based on that of Huhtanen, Rogers & Gall (1952), as modified by King & Smith (1955), was also tested in these experiments. Although the initial mixed cultures utilized up to 90% of the xylan in this medium the pure cultures of Gram-positive cocci and Gram-negative curved rods finally obtained did not grow well and fermented xylan only feebly, which suggests that a medium containing rumen fluid is probably best for initial isolation of the anaerobic rumen bacteria, even if later they will grow in a medium without it.

From the results obtained here it would appear that facultatively anaerobic bacteria do not play a large part in the ruminal fermentation of xylan. The Gram-negative curved rods are among the most active types able to ferment xylan in pure culture and presumably must also be active in the rumen. They were also the predominant bacteria growing in the cultures. The Gram-negative rods isolated in this work all appear to belong to the genus *Butyrivibrio*, but the isolate studied in detail differs in some respects both from the bovine rumen isolate described by Butterworth, Bell & Garvock (1960) and from the bovine rumen type culture of *Butyrivibrio fibrisolvens* (Bryant & Small, 1956). However, of the strains of *Butyrivibrio* so far described no two seem to correspond exactly in properties. No organisms like *Bacteroides amylogenes* (Doetsch *et al.* 1957), which forms an intracellular starch-like polysaccharide, were found. The coccobacillus studied does not compare exactly with any rumen bacterium so far described. Although it has some affinities to *Eubacterium* it does not fit the designation *Eubacterium ruminantium* proposed by Bryant (1959) for some bovine rumen bacteria.

Doetsch *et al.* (1957) found that *Bacteroides amylogenes* formed a high proportion of butyric acid, with smaller amounts of acetic and propionic acids, and a negligible amount of lactic acid by fermentation of xylose. The products of fermentation of xylose by the bacteria described here correspond more to those formed by washed suspensions of mixed rumen bacteria in which acetic is the predominant fatty acid (Heald, 1952; Doetsch, Robinson, Brown & Shaw, 1953; Pazur, Shuey & Georgi, 1958). Formic acid can also be a product of xylose fermentation by mixed rumen bacteria (Heald, 1952). Both the *Butyrivibrio* sp. described here and that previously isolated from the bovine rumen (Butterworth, Bell & Garvock, 1960) produced large proportions of lactic acid in the fermentation of xylose, and the coccobacillus produced succinic acid. Although these acids are not usually found after the fermentation of xylose by mixed rumen bacteria, they could be primary products of xylose breakdown, being further metabolized to propionic acid by bacteria other than xylose fermenting ones (Pazur, Shuey & Georgi, 1958).

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A STAGE-MOUNTED IMMERSION OIL DISPENSER

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THE APPLICATION of immersion oil to the condenser of a microscope and to the slide normally involves the use of some type of dipstick. This is time consuming and wasteful as the size of the drop cannot easily be controlled.

The difficulty can be overcome by the construction of a small stage-mounted oil dispenser (Plate 1). This consists of a suction cup 3·5 cm in diameter, surmounted by a screw to which a spring, 4 cm in length, is attached at an angle of 70° to the horizontal. The spring terminates in a clamp, lined with a short piece of rubber tube, through which is inserted the curved end of a glass reservoir, 11 cm in length. and having a capacity of approximately 5 ml. Filling of the reservoir is done through the upper end which is subsequently closed by a rubber teat.

In use, the suction cup is lightly greased with petroleum jelly and pressed on to the left hand side of the microscope stage. It is then only necessary to bend the dispenser spring towards the condenser lens and apply the desired quantity of oil by slight pressure on the teat.

The dispenser may be used successfully in other microscopical work, as for example in dissections under the stereoscopic microscope, where it is frequently necessary to apply small amounts of water or physiological solutions.

EXPLANATION OF PLATE

PLATE 1. An immersion oil dispenser mounted on a microscope stage.

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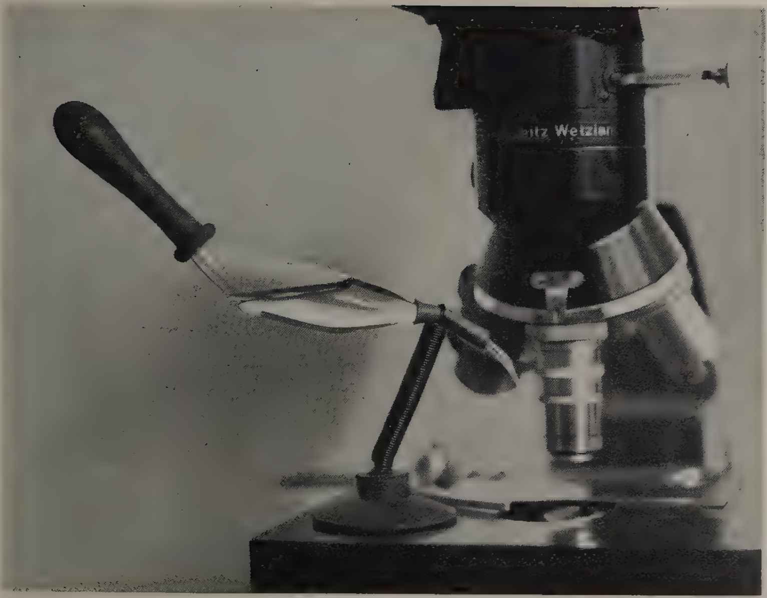

W. A. Smirnoff & Gillian T. Smith — A Stage-mounted Immersion Oil Dispenser. Plate 1

BILLING, E., BAKER, L. A. E., CROSSE, J. E. & GARRETT, C. M. E. (1961). *J. appl. Bact.* **24** (2), 195–211.

CHARACTERISTICS OF ENGLISH ISOLATES OF *ERWINIA AMYLOVORA* (BURRILL) WINSLOW *ET AL.*

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SUMMARY: The characteristics of 38 English isolates of *Erwinia amylovora* were studied independently at East Malling Research Station and at the National Agricultural Advisory Service Laboratories at Wye. The results from both laboratories were similar and indicate considerable physiological homogeneity amongst the isolates. The joint findings are described and compared with those of other authors.

FIREBLIGHT, a bacterial plant disease, has been known in North America for over 150 years and in New Zealand since 1919, but it has only recently been found in this country, first in pears (Crosse, Bennett & Garrett, 1958, 1959, 1960; Lelliott, 1959) and later in other rosaceous plants (Billing *et al.* 1959). The causative organism is *Erwinia amylovora* (Burrill) Winslow *et al.* and the disease is confined mainly to members of the subfamily Pomoideae of the family Rosaceae.

Descriptions of the pathogen in Bergey's *Manual of Determinative Bacteriology* (Breed, Murray & Smith, 1957) and in the *Manual of Plant Pathogens* (Elliott, 1951) are based largely on the observations of Ark (1937), who studied in detail 10 isolates from various sources. Stewart (1913), Reid (1930), Pierstorff (1931), Dowson (1939) and Hildebrand (1954) have also described some of the characters of this organism. When isolates were examined soon after the outbreak in this country, their characters appeared to differ in some respects from the recorded descriptions and a detailed study was therefore made of a number, firstly from pears, and later from other hosts. The study was made independently at two laboratories, at East Malling (EM) and at Wye (W). Different techniques were often employed and the joint findings are compared with those of Ark (1937) and other workers. An account is also given of the sensitivity of the isolates to various phages which were isolated at this time. Characters which were found most useful for diagnostic purposes have been described elsewhere (Billing, Crosse & Garrett, 1960).

MATERIALS AND METHODS

Cultures

Thirty-eight English isolates from a variety of sources were examined in the two laboratories. These consisted of 15 isolated at East Malling in 1958 from infected specimens of pear of the varieties Laxton's Superb (11 isolates), Conference (2), Williams' Bon Chrétien (1), Fondante de Thiriott (1); 14 isolated at Wye in 1958 from Laxton's Superb (11 isolates), Fondante de Thiriott (2) and Catillac (1); 2 isolated in 1959 from a *Crataegus* sp. (1 isolate) and from *Cotoneaster melanocarpa* (1); and 7 isolated in 1960 from a Conference pear (1 isolate), *Sorbus aria* (1), *Sorbus aucuparia* (1), *Cotoneaster salicifolia* (2), *Crataegus* sp. (1) and *Stranvaesia davidii* (1). The isolates were cultured from various parts of infected plants including blossom, fruits, shoots, twigs and branches. Included in the study at both laboratories were two cultures from the National Collection of Plant Pathogenic Bacteria (NCPPB 100 and 311). Cultures were kept on nutrient glycerol agar at 4° (EM) or on Yeastrel agar in screwcapped bottles at room temperature (W). The majority of these cultures are held at the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts. With the majority of tests, all cultures were examined. In cases where only a proportion was tested, this is stated under methods.

Bacteriophages

With one exception, these were obtained from soils of both infected and noninfected orchards by the method described by Crosse & Hingorani (1958). Typical isolates of *Erw. amylovora* were used for isolation and subsequent propagation of the phages. Phage W 23 was isolated from manure, using a culture which was largely noncapsulated and atypical in colony form. Attempts to isolate phages from infected pear material were unsuccessful. In all, 11 phages were isolated and studied.

Morphology and staining

Cultures were grown on Yeastrel agar and examined after 24 hr at 25°. For estimation of size (using an eyepiece micrometer) and for the observation of morphology. motility and capsules, living suspensions were examined using a phase contrast microscope. For capsule demonstration, the wet India ink method recommended by Duguid (1951) was employed. Heat fixed films were stained by Gram's method using the modifications of Hucker (Manual, 1957) and of Nyfeldt (Report, 1949). For the demonstration of flagella, Gray's method was used (Manual, 1957) and also a modification using carbol thionin in place of carbol fuchsin.

Cultural characters

All cultures were incubated at 25° unless otherwise stated.

Colony form. Cultures were grown on Difco nutrient agar containing 5% (w/v) of sucrose (EM), on Yeastrel agar (Yeastrel, 0·3%; Oxoid peptone, 0·5%; New Zealand agar, 1·5%) and on Yeastrel agar containing 5% (w/v) of sucrose (W). About 20 ml of medium were used in a 9 cm diam. Petri dish. Both sucrose agar media were incubated at 25°, but in the case of the plain Yeastrel agar a temperature of 30° was used as colonies were less characteristic at the lower temperature. Colony form was recorded after 2 days; it was most important to have suitable obliquely transmitted light to detect the finer structure of the colonies. In the case of the sucrose agar cultures a further examination was made at 7 days as the development of mucoid growth was sometimes delayed.

Oxygen requirements. These were tested by the shake culture method using 10 ml of nutrient agar plus 5% (w/v) of sucrose (EM) or Yeastrel agar containing 1% (w/v) of glucose (W), in a $6 \times \frac{5}{8}$ in. test tube. Cultures were examined after 7 days.

Optimum pH. This was assessed with 4 cultures (EM) using Difco nutrient broth containing 1% (w/v) of sucrose and adjusted to pH values ranging from 5·2–8·1 by means of phosphate buffers. Turbidity readings were made with a Hilger absorptiometer after 72 hr.

Growth temperatures. Difco nutrient broth (EM) and Yeastrel broth (W) cultures were incubated at temperatures ranging from 6–37°.

Growth factor requirements. These were tested (EM) on 10 isolates of *Erw. amylovora* including NCPPB 100, and also on 2 saprophytic *Erwinia*-like organisms (Billing & Baker, unpublished data). The auxanographic technique of Pontecorvo (1949) was used, with modifications similar to those employed by Bhaskaran & Rowley (1956) for *Vibrio cholerae*. A 50% (w/v) solution of glucose was adjusted to pH 3·0, shaken twice with activated charcoal, filtered through Whatman no. 42 acid-washed paper, adjusted to pH 7·0 and finally sterilized by a momentary steam pressure of 7 lb/in^2. The glucose was then added to an inorganic basal medium (Manual, 1957), containing 1·5% of Oxoid Ionagar no. 2, to give a final concentration of 1%. Growth from a 24 hr nutrient agar slope culture was suspended in 5 ml of glass distilled water, washed 3 times and about 10^7 cells poured into plates with 12 ml of the glucose medium described above. The plates were dried and various growth factors were spotted on the surface of the plates. Choline chloride, nicotinic acid, pantothenic acid, inositol, pyridoxine hydrochloride, thiamine, pimelic acid and β-alanine were employed at concentrations of 1·0 mg/ml, and biotin at 1·0 μg/ml and folic acid and riboflavin as saturated solutions at room temperature. Growth was recorded after 36 hr. All glassware was scrupulously cleaned with chromic acid before use.

Generation time. This was measured at 25° (EM) for one isolate only (P7) which was originally isolated from a canker on a Laxton's Superb pear. The isolate was subcultured daily into Difco nutrient broth at 25° for 8 successive days. An inoculum from the last subculture in the logarithmic phase of growth was transferred to 500 ml flasks containing 100 ml of Difco nutrient broth to give an initial concentration of about 200 cells/ml. The flasks were immersed in a water bath at 25° (±0·1°). Aliquots were withdrawn hourly and viable counts made by plating with 15 ml of nutrient agar containing 5% of sucrose.

Resistance to adverse factors

Heat resistance. This was examined for 2 isolates (EM). Individual 48 hr old cultures in 5% sucrose-nutrient broth were immersed in water baths at 45°, 47·5° and 50°, respectively, and subcultured after 10 min at each temperature.

Antibiotic sensitivity. Sensitivity of 23 isolates and the two type cultures to the following antibiotics was investigated (W): chloramphenicol (100 μg), penicillin (2·5 i.u.), streptomycin (80 μg) and terramycin (10μg). Cultures were tested on Yeastrel agar using 'Sentest' tablets (Evans Medical Supplies Co. Ltd., Liverpool), and incubated at 25° for 2 days.

Sodium chloride tolerance. Cultures were grown in Difco nutrient broth (EM) or Yeast extract glycerol broth (W) containing added NaCl in proportions ranging from 0–7% (w/v). Tubes were examined for turbidity after 2 and 7 days. Eight cultures were tested, including the 2 type cultures.

Bile salt tolerance. Cultures were inoculated into MacConkey's bile salt broth and incubated for 7 days.

Utilization of carbon compounds

All cultures were incubated at 25°.

Mode of utilization of glucose. This was determined by Hugh & Leifson's (1953) method.

Utilization of sugars and related carbon compounds. At EM two different techniques were employed. For the first, an inorganic basal broth medium (Ayers, Rupp & Johnson, 1919) was used with bromocresol purple as indicator (pH 6·8–7·0). Nicotinic acid (0·01% w/v) was included and the test substance, sterilized by filtration, was added to give a final concentration of 1% (w/v) (0·5% in the case of salicin and dulcitol). The medium was tubed in 10 ml quantities and Durham tubes were included. Cultures were incubated for 28 days. About 9 months later, the tests were repeated using an agar disc technique. Sugars were incorporated in the same medium as above plus 2·0% agar; plates were prepared with 15 ml of each sugar medium and dried overnight at 30°. Poured plates of each bacterial isolate, in the same medium but excluding sugars, were prepared the next day using about 10^7 cells/ml of agar. When set, 7 mm discs were cut from the inoculum plates and laid on the surface of the sugar agar. A control disc of unsown agar was also included in each plate. By this method it was possible to test 7 different isolates on one plate of sugar medium. For these tests, sugars were sterilized separately by bringing the autoclave pressure momentarily up to 7 lb/in^2, except in the case of arabinose, xylose, mannose, galactose, sucrose, sorbose and maltose, which were filtered (Rogosa *et al.* 1953).

At W the same basal medium reinforced with nicotinic acid (0·01% w/v) was used but it was solidified with 1·5% of agar. The majority of tests were made on slopes in test tubes, but latterly ½ oz screwcapped bottles were used without any apparent effect on the results. It was found by experience, however, that a more clear cut reaction was obtained in both tubes and bottles if the reaction of the medium was adjusted to pH 6·8 rather than 7·2 or higher. Wherever possible bacteriological grade sugars were used. They were sterilized separately by filtration except in the case of those with low solubilities which were sterilized by autoclaving at 110° for 10 min; they were added aseptically to the basal medium to give a final concentration of 1·0% (w/v).

Utilization of organic acids. Tests were done at EM by the agar disc method using the same basal medium as for sugars, but with bromothymol blue as indicator. The acids were neutralized with NaOH and sterilized separately by momentarily autoclaving at 7 lb/in^2 before incorporating them at a 1% (w/v) concentration in the medium. To overcome possible antagonistic effects (Rhodes, 1959) acids which did not support growth in preliminary tests were retested at a 0·2% (w/v) concentration. At W the latter concentration was used in all tests. A broth base was used in initial tests but gave erratic results and for final tests the acids were incorporated in the same basal medium as used for Simmon's citrate agar, plus 0·01% (w/v) of nicotinic acid, the pH being adjusted to neutrality with NaOH. Cultures were streaked on the surface of poured plates. Citrate utilization was also tested in Koser's citrate broth at both laboratories and on Simmon's citrate agar at EM.

Biochemical tests

All cultures were incubated at 25° unless otherwise stated.

Nitrate reduction. Peptone water (EM) or a semisolid peptone medium containing 0·1% of agar (W) was enriched with 0·1% (w/v) of KNO_3. Cultures were tested with Griess Ilosvay's reagents (using dimethyl-α-naphthylamine) after 1 day and 5 days (EM) or after 3 days (W). Zinc dust was added to all cultures giving a negative reaction to ensure that nitrate remained and had not been decomposed beyond the nitrite stage.

Indole production. Cultures were grown in Oxoid peptone water containing 0·5% of Difco yeast extract for 2 and 5 days (EM) and tested with Ehrlich's reagent; or in Difco tryptone water for 3 days (W) and tested by the methods of Kovacs and Gnezda (Manual, 1957).

Methyl red (MR) and Voges-Proskauer (V-P) tests. Cultures were grown in Oxoid glucose phosphate broth plus 0·5% of Difco yeast extract and tested after 2, 3 and 5 days (EM) or after 3 days (W). For the V-P test for acetoin, a pinch of creatine and 5 ml of 40% NaOH were added to 5 ml of culture. After thorough shaking, tubes were allowed to stand and a final reading taken after 3 hr. Later, the V-P test was repeated on several isolates at both laboratories; at EM a medium consisting of: peptone, 0·5%; glucose, 0·5%; and K_2HPO_4, 0·5% was used, and Barritt's (1936) α-naphthol method was employed to test for acetoin after 3 and 5 days. At W tests were repeated using the original method but incubation was prolonged for 7 days.

Hydrogen sulphide production. Lead acetate strips suspended over peptone water cultures were used (Manual, 1957). Incubation was for 4 days (EM) or 7 days (W).

Ammonia production. Cultures were grown in 1% peptone water plus 0·5% of Difco yeast extract for 2 and 5 days (EM) or in 0·25% peptone water for 5 days (W), then tested with Nessler's reagent.

Arginine breakdown. Cultures were grown for 14 days in the Manchester medium described by Sherris, Shoesmith, Parker & Breckon (1959) and tested by the method recommended by these authors.

Gluconate test. The modified Hayne's medium of Shaw & Clarke (1955) was used. Tests were made after 2 and 6 days (EM) and after 4 days (W) with Benedict's qualitative reagent.

Phenylalanine and malonate tests. The combined medium and the method of Shaw & Clarke (1955) were used. Incubation was for 2 and 5 days.

Aesculin hydrolysis. This was tested by Sneath's method (1956), using either a liquid medium (EM) or an agar medium as slopes in screwcapped bottles (W). Incubation was for 14 days.

Casein hydrolysis. At EM cultures were grown for 7 days on the following medium (Hayward, pers. comm.): casein (BDH light white soluble), 1·0%; K_2HPO_4, 0·1%; $MgSO_4.7H_2O$, 0·1%; NaCl, 0·1%; Oxoid peptone, 0·5%; and agar 2%. At W, 1 ml of skim milk was added to 10 ml of Yeastrel agar. Cultures were spotted on the dried surface of the plates and incubated for 5 days.

Starch hydrolysis. Cultures were streaked on 0·2% starch-nutrient agar and incubated for 8 days (EM) or on 1% starch-Yeastrel agar and incubated for 5 days (W).

Gelatin hydrolysis. Stab cultures were made in nutrient gelatin at both laboratories; also in plain gelatin (EM). Incubation was for 20 days at 20° (EM) or for 21 days at 22° (W).

Liquefaction of pectate gel. Cultures were incubated for 4 days on Luckhurst's (1953) sodium pectate-calcium agar medium.

Fat hydrolysis. Olive oil in a concentration of 2% (v/v) was incorporated in 10 ml of Yeastrel agar with neutral red (0·1 ml of a 1·0% solution) as indicator. Cultures were spotted on the surface and incubated for 5 days (W).

Hydrolysis of tributyrin. Fifteen cultures were tested by Rhodes' (1959) method. Cultures were incubated for 7 days (EM).

Hydrolysis of Tween 80. Fifteen cultures were tested by Sierra's (1957) method (T.B. culture grade of Tween 80) and the cultures incubated for 7 days (EM).

Catalase activity. A loopful of growth from a 24 hr culture on nutrient agar plus 2% of glycerol was emulsified in 2 ml of water and a drop of 100 vol H_2O_2 added (EM). Alternatively, 1 ml of 20 vol H_2O_2 was pipetted on to a 24 hr culture on nutrient agar and observed for the evolution of gas bubbles (W).

Oxidase activity. Nutrient agar cultures were tested by Kovacs' (1956) method (EM) and broth cultures by Graby & Hadley's (1957) method (W), in both cases after incubation for 48 hr.

Urease activity. Cultures were grown on Christensen's (1946) urea medium for 7 days and examined for the production of an alkaline reaction.

Lecithinase activity. At EM the reaction on egg yolk was tested by the method of Macfarlane, Oakley & Anderson (1941). At W, egg yolk plates were prepared by adding 1 ml of an egg yolk suspension to Yeastrel agar containing 1% of NaCl (Billing & Luckhurst, 1959). Spot cultures were made on the dried surface and the plates were incubated for 5 days.

Tyrosinase activity. This was tested on 15 isolates at EM on the medium devised by Hayward (pers. comm.): sucrose, 0·5%; casein hydrolysate (Oxoid), 1·0%; K_2HPO_4, 0·05%; $MgSO_4.7H_2O$, 0·0125%; L-tyrosine (BDH), 0·1%; agar, 2·0%. Cultures were streaked on agar plates and observed for browning of the medium after incubation for 4 days.

Litmus milk. This was prepared from Oxoid granules (EM) or Oxoid skim milk powder with litmus solution added separately (W). Incubation was for 14 days.

Pathogenicity tests

These have been described in detail in a previous paper (Billing *et al.* 1960); immature pear fruits, either whole or sliced, were used. After inoculation they were kept at 25° under moist conditions and observed daily for the production of a milky white ooze.

Phage sensitivity tests

These have also been described previously (Billing *et al.* 1960). The medium contained Difco yeast extract, 0·3%; Oxoid peptone, 0·5%; glycerol, 2·0%; and New Zealand agar, 1·0%. For the test, 1 ml of a 24 hr broth culture or a light suspension was added to 12–15 ml of melted, cooled agar and a poured plate prepared. Surface drying of the plate was usually done, but was not always found to be essential. Phages were streaked or spotted on the surface, the former method being used when plaque morphology was important. Plates were incubated at 25° for 24 hr.

RESULTS

Morphology and staining

Cells were small, ovoid or rod shaped, and occurred singly or in pairs or groups; occasional long rods or filaments were also seen. A colonial variant of one of the type cultures, 311, was predominantly filamentous. Most cultures showed varying proportions of all these forms, but some were either predominantly coccoid or rod shaped. The small size of the cells made accurate measurement impossible, but living cells examined with a phase contrast microscope were mainly about 0·8 μ in diameter and, apart from filamentous forms, varied in length from 1·0–3·0 μ, the most common length being between 1·2 and 1·8 μ. Heat fixed stained cells were only about 0·5μ in diameter and about 0·8–1·2 μ long.

All cultures contained motile cells but under the conditions of the test there were variations between cultures in this respect, some showing many actively motile cells, others only a few. There was no obvious correlation between morphology or degree of motility and any other character, though this was not investigated in detail. The majority of cultures were capsulated, but varied in their degree of capsulation and there was a definite relationship between this character, colony form and phage sensitivity (Billing, 1960). Both type cultures showed a proportion of capsulated cells. Some difficulty was experienced with Gram's stain, mainly because of low intensity of staining, but there was also a tendency to resist decolorization. Using Hucker's or Nyfeldt's method unmodified, cells often appeared pale violet or pale red. No improvement in staining was observed when cultures were incubated at 30° instead of 25° or for 48 hr instead of 24 hr. When acetone was used for decolorization and 1 in 10 carbol fuchsin as counterstain, a more definite reaction was obtained. With this modification all except 3 of the 20 cultures examined were predominantly Gram-negative, though a few pale violet cells were usually observed. The remaining 3 cultures were predominantly violet, with a small proportion of red cells, but the intensity of the violet stain was low compared with a definite Gram-positive *Corynebacterium* sp. which was examined for comparison. Again there was no obvious correlation between staining reaction and any other character.

All of the 17 cultures examined by Gray's method were flagellated, the majority showing 1–5 flagella. Although the carbol thionin method gave a less clear background, there was better staining of the flagella and in such preparations up to 8 flagella/cell were seen.

Cultural characters

Colony form. On 5% sucrose agar most cultures produced domed, mucoid colonies, varying from 3 to 4 mm in diameter after 3 days. They were cream coloured and granular in structure; some developed a translucent edge and colonies tended to coalesce on further incubation (cf. Ark, 1937). Two cultures were not mucoid on this medium and the colonies were only 1–2 mm in diameter; four others were less mucoid than normal and showed a different structure, and the colonies did not exceed 3 mm in diameter. These cultures were atypical on Yeastrel agar and possessed relatively few capsulated cells when examined microscopically.

On Yeastrel agar, colonies were not distinctive at 25°, but at 30° those cultures giving typical domed colonies on sucrose agar developed a characteristic marking. Colonies were about 1·0 mm in diameter after 2 days, were raised, entire, opaque, cream coloured and had a slightly granular structure. Markings, resembling cross hatching, made them readily recognizable, but this was only seen when colonies were observed with a hand lens by oblique transmitted light (Billing *et al.* 1960). The atypical cultures mentioned above were slightly smaller and more translucent and grey; on further incubation they tended to show a dense centre with a more transparent edge. The differences in colony form were not absolute and intermediate forms with striations or sectors were observed in some cultures. Of the 23 cultures examined at Wye, 10 were atypical or intermediate in colony form. After holding in stock for several months it was usual to find that a mixture of typical and atypical colonies was obtained on restreaking from an originally typical culture. Occasionally when a stored atypical culture was streaked a few typical colonies were observed, but this was rare. The colonial character of cultures remained fairly stable over a period of 2 years, though restreaking and reselection of typical colonies was found necessary in some cases. Both the type cultures showed considerable colonial variation on initial cultivation from freeze dried preparations. From one, NCPPB 100, two stable colony forms were obtained which resembled those described above; with the other, NCPPB 311, stable cultures were obtained of a featureless colony form and a granular one, both of which showed a proportion of capsulated cells. The latter variant gave granular growth in broth, but the agar colonies were butyrous in consistency and gave a stable suspension in saline. No rough forms (Ark, 1937) were observed with any cultures or on isolation plates. In the latter case no special search was made for them and their occurrence may have been overlooked.

Oxygen requirements. The 17 isolates and the two type cultures tested all behaved in the same way. Growth was profuse at the top of the shake tube, but submerged growth was poor and colonies could be detected only with a hand lens.

Optimum pH. The 4 cultures grew well over the range tested (pH 5·2–8·1) with maximum growth at approximately pH 6·0.

Growth temperatures. No growth was observed at 3–5° or at 37°. Growth was slow at 6–8°, but only one isolate failed to grow in 7 days. At 22°, 25°, 27·5° and 30°, good growth was obtained within 48 hr. Turbidity was uniform at the higher temperatures but inclined to be granular at 22°. The optimum temperature as indicated by turbidity measurements at 48 hr was 25–27·5°.

Growth factors. All isolates of *Erw. amylovora* tested required nicotinic acid for growth; there was no response to any other growth factor. This agrees with the findings of Starr & Mandel (1950). The two saprophytic *Erwinia*-like organisms tested were not exacting, growth occurring uniformly over the whole plate.

Generation time. Over a period of 6 hr at 25° the mean generation times of P7 on three separate occasions were 74·9, 70·3, and 72·1 min, respectively. This compares with generation times at 30° varying from 71–94 min with different strains of *Erw. amylovora* which were reported by Hildebrand (1938).

Heat resistance. With the two cultures tested, survivors were detected after 10 min at 45° and 47·5° but not at 50°.

Antibiotic sensitivity. Under the conditions of testing, all cultures were sensitive to chloramphenicol, streptomycin and terramycin but were resistant to penicillin.

Sodium chloride tolerance. Growth was not completely prevented until the NaCl concentration was 6–7%, but it was retarded at concentrations above 2%.

Bile salt tolerance. All cultures grew in MacConkey's broth, turbidity usually being apparent within 48 hr.

Utilization of carbon compounds

Mode of utilization of glucose. Erwinia ananas was included for comparison in these tests and gave a distinct fermentative type of reaction similar to other *Erwinia* spp. (Hayward, pers. comm.). *Pseudomonas mors-prunorum* and *Ps. syringae*, also included, gave an oxidative type of reaction characteristic of this genus. Results with *Erw. amylovora* were less clear cut. All of the 38 isolates tested produced acid near the surface of the unsealed medium after 2 days; the reaction in the sealed medium was similar, but slower, and an acid reaction was barely perceptible after 3 days in most cases. Even after 6 days the reaction tended to be localized near the surface and was seldom as strong as in the unsealed medium. A complete acid reaction was eventually obtained in both sealed and unsealed media after 2 weeks. Sealed tubes inoculated with the *Pseudomonas* spp. were still unchanged after this period.

The indeterminate behaviour of *Erw. amylovora* in Hugh & Leifson's (1953) medium appears to be characteristic of this organism, and the results obviously reflect its strongly aerobic nature as shown by the shake culture experiments. Sutton & Starr (1959) have shown that suspensions of non-growing cells of *Erw. amylovora* will ferment glucose and have commented on the difficulty of studying fermentation with growing cultures owing to the complexity of the medium necessary to support growth in the absence of air.

Utilization of sugars and related compounds. There were variations in the rate at which some compounds were utilized, and one culture is described later in more detail which was slow to grow on all the sugar media except that containing glucose. With most compounds, all isolates gave the same final reaction, and the results were consistent irrespective of the method of test used or of the length of time the isolates had been held in stock since original isolation. The exceptions were xylose, maltose and rhamnose, where isolates varied, and the results of different tests were inconsistent. Cellobiose gave consistent results but isolates varied in their reaction.

Growth and acid production were more rapid on agar than in broth media; there was no evidence of gas production in the latter. The results are summarized in Table 1. Control inoculated media containing no added carbon source were invariably negative.

Table 1. *The utilization of sugars and related compounds by English isolates of* Erwinia amylovora

Acid production from given substrates			
Rapid*	Slow*	Negative	Variable
Arabinose	Cellobiose†	Sorbose	Xylose
Fructose	Salicin	Lactose	Rhamnose
Galactose	Inositol	Raffinose	Maltose
Glucose	Sorbitol§	Glycogen	
Mannose		Starch	
Sucrose		Inulin	
Trehalose		Dextrin	
Glycerol		α-Methylglucoside	
Mannitol			
Sorbitol‡		Dulcitol	

* Rapid: agar, 2–3 days; broth, 5–10 days. Slow: agar, 4–14 days; broth, >16 days. †Two cultures and one type culture (NCPPB 311) were negative.

‡ In agar culture only. § In broth culture only.

Maltose. The experience at both laboratories was that different results were obtained on different occasions of testing. At EM, 6 out of 9 cultures were positive in an initial broth test, but when tested later by the agar disc method, all tests were negative. At W, 6 out of 19 cultures tested were positive in 3–5 days on maltose agar slopes, but when the tests were repeated on these cultures a year later on the same medium, none was positive. This suggested that ability to attack maltose might be a character which was readily lost after initial isolation, but tests on 25 freshly isolated cultures were all negative. The test was repeated 2 years later on 25 cultures, and on this occasion 3 cultures, including 2 which had previously been positive and 1 which had been negative, gave a positive reaction. The 2 type cultures were consistently negative. In both initial and repeat tests there was a close correlation between acid production from maltose, xylose and rhamnose.

Xylose. In initial tests at both laboratories only a proportion of isolates gave a positive reaction. Using the agar disc method 9 months later (EM), all isolates were positive. In repeat tests after 2 years at W, all isolates including the 2 type cultures produced a very weak reaction in 7–21 days, except for the 3 maltose positive cultures which produced a strong reaction in 5–7 days.

Rhamnose. At EM variable results were obtained in initial broth tests, but repeat tests using the agar disc method were all negative. At W some isolates were positive in initial tests and in repeat tests 2 years later, the 3 maltose positive cultures and 2 others were positive though acid production was delayed. The two type cultures were consistently negative. Control inoculated media containing no added carbon source were invariably negative.

Slow growing isolate. One isolate from a *Cotoneaster* sp. grew and produced acid very slowly on most sugar media. Acid production on glucose slopes was nearly normal, a positive reaction being obtained in 3–4 days, but with all other sugars and related compounds which were attacked acid production was delayed for 12 to 21 days. Often only a few colonies appeared on the slope, suggesting the selection of a variant from a population which was largely incapable of utilizing that carbon source. The normal growth on the glucose medium suggested that it was not due to a growth factor deficiency. A similar slow utilization of sucrose was observed with 2 additional cultures during routine diagnoses.

Utilization of organic acids. At EM, the following acids were utilized by all isolates: L-malic, fumaric, succinic and citric, the latter only at the lower concentration 0·2%. No growth occurred at a concentration of either 1% or 0·2% with acetic, formic, lactic, malonic, maleic, oxalic, propionic and tartaric acids. Similar results were obtained at W but growth on citrate agar was weak, even at the 0·2% concentration, and some of the isolates grew poorly on all the media.

Biochemical tests

Negative results were obtained with all cultures in the following tests: the production of oxidase, urease, tyrosinase, nitratase, phenylalanine deaminase, indole, and a methyl red reaction; hydrolysis of arginine, casein, starch, fat, lecithin, tributyrin and Tween 80; liquefaction of pectate gel, and utilization of malonate.

Catalase activity. All cultures gave a strong positive reaction.

Voges-Proskauer test. Initially when tested after 2, 3 or 5 days, all isolates were V-P negative. When incubation was extended to 7 days, however, weak positive reactions were observed with the majority of cultures. At EM, 9 isolates (including the 2 type cultures) were tested using Barritt's (1936) method. Of these a proportion were positive after 3 days, but after 5 days incubation all gave a positive reaction except NCPPB 311.

Hydrogen sulphide production. All cultures were negative in this test except NCPPB 311, which was weakly positive.

Ammonia production. At EM all cultures tested gave a negative result with the exception of NCPPB 311. At W all cultures, including the 2 type cultures, gave weakly positive results.

Gluconate test. Variable results were obtained at both laboratories. Only 2 isolates gave a strongly positive reaction (a strong orange precipitate) when tested with Benedict's reagent after incubation for 3 or 4 days, but 6 further isolates gave a green-orange colour. Except for 3 isolates which showed no reaction after this period, the remainder, including the 2 type cultures, gave colour changes varying from grey to yellow-green with very little precipitate. Two of the 3 negative isolates were retested at 6 days, when a moderate reaction was observed.

Aesculin hydrolysis. Variable results were obtained at both laboratories. Seven isolates gave a definite positive reaction in 4–7 days, 10 were positive after 13 days and 5 caused no change in the medium during this period. The remaining isolates caused loss of blue fluorescence and darkening of the medium but there was no distinct black discoloration.

Of the 2 type cultures, NCPPB 100 gave a positive and 311 a negative reaction after 13 days. Further incubation up to 21 days of a proportion of negative or doubtful cultures produced little further change.

Gelatin hydrolysis. All except one culture (a noncapsulated variant of an originally typical culture) caused crateriform liquefaction of nutrient gelatin within 28 days. There was some correlation between rate of liquefaction and colony form. With the 2 type cultures and all cultures showing typical colonies liquefaction was first apparent in 2–3 days. With cultures which were atypical in colony form, the time when liquefaction was first observed varied from 3–21 days. Plain gelatin without added nutrients was not liquefied by the 12 isolates which were tested.

Litmus milk. All cultures slowly produced an alkaline reaction and partial or almost complete reduction of the litmus. Slight digestion was observed at the surface with the majority of cultures, extending to a maximum depth of 4 mm after 21 days.

Pathogenicity tests

All except one isolate produced ooze in small immature pear fruits or slices. The negative isolate was one of the group which were atypical in colony form. Other members of this latter group were generally slower to produce ooze, some taking 3–4 days in contrast to 2 days which was characteristic of typical cultures. One type culture, NCPPB 311, was also negative in this test when examined about 2 years after culturing from the freeze dried state, although originally it had been positive. Some isolates were inoculated into pear branches, shoots and blossoms and were highly virulent (Crosse *et al.* 1960).

Phage sensitivity tests

All cultures which were typical in colony form were sensitive to phages EM3, EM4a, EMW1, W4S and W4L. The plaque morphology of W4S and W4L has been described and illustrated elsewhere (Billing *et al.* 1960). Some of the atypical cultures were also sensitive to these phages, but in general they showed greater sensitivity to phage W23. Typical cultures were relatively insensitive to the latter phage. The relationship between capsulation and phage sensitivity has been described in detail elsewhere (Billing, 1960). Briefly, it was observed that cultures possessing a high degree of capsulation and producing typical colonies gave large clear plaques with haloes with phage W4L, small clear plaques with haloes with phage W4S and little or no reaction with phage W23. In contrast, cultures with a low degree of capsulation and atypical colonies showed little or no reaction with phages W4L and W4S, but gave small slightly turbid plaques without haloes with phage W23. Cultures with an intermediate degree of capsulation gave turbid plaques with all 3 phages. It was thought that the halo formation with phages W4L and W4S might be due to a capsular hydrolyzing enzyme.

The two colony forms of NCPPB 100 behaved according to the pattern described above, but NCPPB 311, though sensitive to W4S and W4L, gave only small plaques with W4L; reaction with W23 was weak.

These phages were tested on other *Erwinia* spp. and similar organisms. Phages W4S and W4L caused some lysis of *Erwinia lathyri* (NCPPB 202) and a trace reaction with *Erw. ananas* (NCPPB 548). Some non-pathogenic *Erwinia*-like organisms isolated from a variety of plants have also exhibited varying degrees of sensitivity to these phages (Billing & Baker, unpublished data). The following species were found to be insensitive: *Agrobacterium tumefaciens* (NCPPB 4), *Agr. rhizogenes* (NCPPB 5), *Agr. pseudotsugae* (NCPPB 180), *Erwinia rhapontica* (NCPPB 139), *Erw. nimipressularis* (NCPPB 440), *Erw. salicis* (NCPPB 447), *Erw. carotovora* (NCPPB 468), *Erw. chrysanthemi* (NCPPB 513), *Erw. aroidae* (NCPPB 546) and *Erw. nigrifluens* (NCPPB 566).

Phages EM1–EM6, inclusive, had a similar host range, i.e. they reacted with *Erw. amylovora*, *Erw. ananas* and *Erw. laythri*, but with no other species of *Erwinia* or *Agrobacterium*. They also failed to react with isolates of *Escherichia coli*, *E. freundii* and *Klebsiella aerogenes*.

Temperature had some effect on phage activity. W4L and W23 were active at both 25° and 30°, but W4S showed little or no activity at the higher temperature.

DISCUSSION

An outstanding feature of this study was the uniformity of character of the isolates examined, although they were obtained from different hosts and from a variety of localities in South East England. If the results with xylose, rhamnose and maltose, which were inconsistent, are ignored, the main physiological variation occurred in respect of gluconate oxidation and aesculin hydrolysis and even here the differences in activity between isolates was quantitative rather than qualitative. Colony form and phage sensitivity also varied, but these were associated with the degree of capsulation and appeared unrelated to the origin of the isolates or to their biochemical activity. Ark (1937) and Hildebrand (1954) observed greater variation among their isolates than was found in the present study, but how far this difference is due to the methods of test or to other factors is uncertain. Elrod (1941*a*) found *Erw. amylovora* to be an exceedingly homogeneous species serologically, in marked contrast to isolates of the *Erw. carotovora* group (Elrod, 1941*b*).

It might be argued that the uniformity of the English isolates was due to the introduction of a single strain of *Erw. amylovora* into this country. While this may be true, it should be pointed out that the two available type cultures, both originaly, from North America, were almost identical in character. One of these, NCPPB 1010 has been in culture since 1937. This suggests that most of the physiological characters we have observed may be fairly stable. Hildebrand (1954) has described *Erw. amylovora* as relatively unstable, but he was referring to loss of virulence in culture; he stressed that virulence showed practically no correlation with physiological behaviour.

The study of the host range of the *Erw. amylovora* phages suggests that *Erw. amylovora*, *Erw. lathyri*, *Erw. ananas* and the *Erwinia*-like organisms from various plants (Billing & Baker, unpublished data) have some affinities not shared by other *Erwinia* spp. This is undoubtedly true of the two yellow chromogenic forms *Erw. lathyri* and *Erw. ananas*, which Graham (1958) found to be biochemically and physiologically indistinguishable.

In comparison with other *Erwinia* spp., *Erw. amylovora* does not show pronounced metabolic activity. Its weak fermentative power is reflected in Hugh & Leifson's test, and the gluconate, MR and V-P tests. Even under aerobic conditions, acid production from sugars is slow in broth, and surface culture on agar media is recommended for these, and other, biochemical tests.

Although different testing methods were often employed, and the cultures were not maintained under the same conditions, no major differences in character were observed by the two collaborating laboratories. The joint results are not, however, identical with those of Hildebrand (1954), Ark (1937) and Dowson (1939) and do not therefore accord in all respects with the lists of characters quoted in the Manuals of Bergey (1957) and Elliott (1951) who rely largely on the results of the latter two authors.

Elliott (1951) stated that *Erw. amylovora* is not capsulated, which does not accord with our findings. Variation in colony form was observed by both Ark (1937) and Sutton & Starr (1959) and in most respects their descriptions agree with ours, though we did not observe the rough form described by Ark.

Ark (1937) and Hildebrand (1954) are the only workers who have given detailed descriptions of the characters of a number of isolates of *Erw. amylovora* and some of the differences between our findings and theirs with regard to the utilization of carbon compounds are summarized in Table 2. The full significance of these discrepancies cannot be assessed, but they undoubtedly reflect, in part at least, differences in the test methods employed. In the V-P test, Ark's negative result may have been due to a medium factor or to the failure, as in some of our tests, to use a method sufficiently sensitive to detect acetoin.

Table 2. *The differences* between English isolates of* Erwinia amylovora *and 2 type cultures, and those studied by Ark* (1937) *and Hildebrand* (1954), *in ability to utilize various carbon compounds*

Carbon compound	Ark (1937) (10 cultures)	Hildebrand (1954) (136 cultures)	English isolates (38 cultures)	Type cultures (2 cultures)
Xylose	—	—	V	V
Rhamnose	—	V	V	—
Galactose	V	A	A	A
Lactose	V	V	—	—
Glycerol	V	V	A	A
Mannitol	V	A	A	A
Maltose	A	V	V	—
Raffinose	A	V	—	—
Dextrin	A	A	—	—
Lactate	V	V	+	—
Succinate	V	Alk	—	—

* A, acid; Alk, alkaline; V, variable results; —, negative.

We also differ from these authors on another point of special interest. Ark observed that his more virulent strains were slow to attack gelatin, 2 being completely negative in this test. All Hildebrand's isolates liquefied gelatin, avirulent cultures being as active as, or more active than, the virulent ones. In our experience those isolates which attacked gelatin slowly were also slow to produce ooze in pear fruits and were also characterized by their low degree of capsulation. We are in full agreement with Ark and Hildebrand with regard to the following characters which may, therefore, be regarded as stable for this organism, independent of origin or of the test method used. Positive characters: acid from glucose, sucrose, arabinose, fructose and salicin; citrate and malate utilized. Negative characters: no acid from dulcitol; malonate and oxalate not utilized; nitrate not reduced to nitrite; urea and starch not hydrolyzed; indole not produced.

A full summary of all the characters of the isolates studied in this investigation is given below.

A description of 38 *English isolates of* Erwinia amylovora

Small rods, approximately 0·8 to 1·0 by 3·0 μ (living cells), occurring singly, in pairs or in groups. Small capsules. Motile by means of peritrichous flagella. Gram-negative or variable.

Colonies on 5% sucrose agar domed and mucoid: on Yeastrel agar, opaque, cream, circular with characteristic markings at 30°; some isolates grey and unmarked.

Aerobic; poor growth under anaerobic conditions. Growth range 6–30°, optimum about 25°. Grow with ammonium salts as sole source of nitrogen, but nicotinic acid an essential growth factor. Optimum pH 6·0. Destroyed by heating for 10 min at 50° (limited tests only). Sensitive to chloramphenicol, streptomycin and terramycin but not to penicillin. Growth retarded by salt concentrations above 2%. Grow in bile salt broth.

Acid production from glucose rapid under aerobic but slow under anaerobic conditions. Acid (but no gas) produced rapidly from the following compounds when supplied as sole carbon sources in a chemically defined medium (pH 6·8–7·0) containing 0·01% of nicotinic acid: arabinose, fructose, galactose, glucose, mannose, sucrose, trehalose, glycerol, mannitol and sorbitol; slow acid production from cellobiose, salicin and inositol. No acid from sorbose, lactose, raffinose, glycogen, inulin, dextrin, starch, α-methyl glucoside or dulcitol. Acid production from xylose, rhamnose and maltose variable. Organic acids normally utilized with the production of alkali: l-malic, fumaric, succinic and citric (the latter at 0·2% but not at 1·0% concentration); acetic, formic, lactic, malonic, maleic, oxalic, propionic and tartaric acids not utilized.

Catalase positive. Gelatin liquefied. Acetoin production weak (detection depending on test method). Oxidation of gluconate and hydrolysis of aesculin variable. Ammonia production weak or negative. Litmus milk slowly turned alkaline, slight digestion, litmus reduced. Oxidase, urease, tyrosinase and phenylalanine deaminase negative. Nitrate not reduced to nitrite. Hydrogen sulphide not produced. Indole and methyl red negative. Casein, starch, fat, lecithin, tributyrin, Tween 80 and arginine not hydrolyzed. Pectate gel not liquefied. Malonate not utilized.

Produce milky ooze in immature pears in 2–4 days. Sensitivity to phages depends on degree of capsulation.

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INVESTIGATIONS ON THE USE OF THE ROLL TUBE METHOD FOR COUNTING *ESCHERICHIA COLI* I IN WATER

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SUMMARY: The roll tube method was used for the enumeration of *Escherichia coli* I in water and was found to be subject to less variation than the accepted method. Different media were compared and it was found that MacConkey's broth containing 3% of agar gave the best results. Pre-incubation at 37° for 2 hr improved the selectivity and gave higher colony counts.

The standard method for determining the number of coli-aerogenes organisms in a sample of water described in the Ministry of Health's Report (Report, 1957*a*) is subject to large errors because of the small number of tubes examined at each dilution and because of the large dilution factor involved. Halvorson & Ziegler (1933) stated that when five tubes are used for each of three tenfold dilutions of water the most probable number of coli-aerogenes organisms present, indicated by the probability tables, may be too low by 70% of the real value or too high by 260%. This variation makes the technique unsuitable for the analysis of water samples when small differences between samples are important. Small differences are important in investigations such as those at present being carried out at this Institute on the changes of bacterial densities with depth in 'oxidation ponds', where the biological self purification processes in natural waters are exploited to achieve the economical purification of sewage.

In this paper the results are reported of an investigation designed to compare the most probable number (MPN) technique with the roll tube method, and the selectivity of different media and the influence of pre-incubation time on the count of *Escherichia coli* I obtained by the roll tube method.

Materials and Methods

Apparatus. An Astell roll tube apparatus as described in Report (1957*b*) was used.

Media. The following Difco preparations were used for selective counting: brilliant green-bile (2%) broth; brilliant green-bile agar; MacConkey's agar; and MacConkey's broth. For typing, the following Difco products were used: MacConkey's broth; SIM medium; Koser's citrate medium; nutrient gelatin; and M.R.-V.P. medium.

In all cases where agar was used it was found necessary to increase the final agar concentration to 3%; otherwise, the media did not solidify sufficiently to cling to the sides of the roll tubes.

Enumeration and typing of E. coli. Volumes of 5 ml of the appropriate agar medium were added to the roll tubes and then sterilized. Before use the medium was melted and kept in a thermostatistically controlled water bath at 48°. The water sample to be counted was suitably diluted in quarter-strength Ringer's solution and 0·5 ml amounts pipetted into triplicate tubes, which were rolled and incubated at 44·5°. The colonies were counted after 48 hr. The dilutions were made by transferring 10 ml of the sample into 90 ml of sterile quarter-strength Ringer's solution.

For typing, all the colonies or an unbiased selection of the colonies in the roll tubes were isolated and purified by streaking on MacConkey's broth containing 1·5% of agar. Single colonies were then picked and transferred to a nutrient broth, which was then used as an inoculum for the differential media.

The typing was done according to the system outlined in Table 1, which is a compilation of the data used by Wilson *et al.* (1935), Report (1956) and Breed, Murray & Smith (1957).

Table 1. *Key used for typing the coli-aerogenes bacteria*

Identity	Results* for tests						
	MacConkey's broth at 45°	Indole formation	M.R.† reaction	V-P‡ reaction	Citrate utilization	Gelatin liquefaction	H_2S production
Escherichia coli I	+	+	+	–	–	–	–
E. coli II	–	–	+	–	–	–	–
E. coli III	–	+	+	–	–	–	–
Citrobacter freundii I	–	–	+	–	+	–	+
Citr. freundii II	–	+	+	–	+	–	+
Klebsiella aerogenes I	–	–	–	+	+	–	–
K. aerogenes II	–	+	–	+	+	–	–
K. cloacae	–	–	–	+	+	+	–
Irregular II	+	–	+	–	–	–	
Irregular III		–	+	–	–	+	
Irregular IV	–	–	+	–	+	+	
Irregular V	–	–	–	+	–	–	
Irregular VI	+	–	–	+	+	–	
Irregular VII	–	+	–	–	+	–	
Irregular VIII	–	–	–	–	–	–	–

* +, positive reaction; –, negative reaction. † M.R., methyl red. ‡ V-P, Voges-Proskauer test.

RESULTS

Comparison between the roll tube and MPN techniques. A sample of settled sewage water was thoroughly agitated in a macerator and then divided into 8 portions. Each of these portions was treated as a separate sample and the same media (MacConkey's broth and MacConkey's agar) were used. The results from this comparison are shown in Table 2. It is clear that the coefficient of variation of the roll tube counts was much lower than that of the MPN counts, and that on the same medium the roll tubes gave an average count from 3 to 12 times lower than the average MPN.

The use of different coli-aerogenes enumeration media in the roll tube. Difficulties were encountered in counting colonies which developed on MacConkey's agar and on brilliant green-bile agar because of the opaqueness of these media. MacConkey's broth and brilliant green-bile (2%) broth, which were used for the MPN, were the most suitable for counting purposes. These broths were used with the addition of 3% of agar.

A comparison of the colony counts on these media showed that on MacConkey's agar they were 4 to 5 times higher than on brilliant green-bile (2%) agar, the means of 150 comparisons being 26,347 and 5,687, respectively.

Table 2. *A comparison of the bacterial numbers obtained from eight replicate samples of the same water by the MPN and roll tube methods*

Sample no.		Viable counts* (/ml) by	
		MPN method	Roll tube method
1		79,000	11,800
2		79,000	11,300
3		79,000	11,600
4		31,000	11,300
5		33,000	11,600
6		46,000	10,000
7		130,000	10,900
8		49,000	9,900
	Arithmetic mean	65,750	11,050
	Coefficient of variation	50·14%	6·6%

* Counts done in MacConkey's medium at 44·5°.

In typing the bacteria which developed from the same water samples very little difference was noticed in the selectivity of the two media for *E. coli* (Table 3).

Table 3. *The incidence of different coli-aerogenes organisms developing in MacConkey's agar and brilliant green-bile* (2%) *agar in roll tubes*

Type	Incidence (%) of types from	
	MacConkey's agar	Brilliant green-bile agar
Escherichia coli I	79	79
E. coli III	2	0
Irregular II	6	7
Irregular VI	4	0
Irregular VIII	4	7
Klebsiella aerogenes I	1	0
Not typed	4	7

These results indicate that MacConkey's agar is preferable to brilliant green-bile (2%) agar because, although the two media showed the same selectivity for *E. coli* I, the former gave much higher counts.

Pre-incubation. A further attempt was made to increase the numbers of *E. coli* I without changing the selectivity of the medium. Childs & Allen (1953) described a 'resuscitation' method for the estimation of faecal *E. coli* in water. On the basis of these findings and the well known fact that more colonies develop at 37° than at

44·5°, the following experiment was done. Four sets of roll tubes of MacConkey's broth containing 3% of agar were inoculated with 'oxidation pond' water. The first set was incubated at 44·5°; the second, third and fourth sets were given pre-incubation periods at 37° of 2, 4 and 6 hr, respectively, before incubating at 44·5°.

Table 4. *The effect of different pre-incubation periods at 37° on the counts on MacConkey's agar incubated at 44·5°*

Sample no.	Colony counts at 44·5° after pre-incubation at 37° for (hr)			
	0	2	4	6
1	330	2,700	3,400	15,500
2	620	42,300	2,540	*
3	4,080	52,200	1,220	3,000
4	532	9,400	4,520	9,900
5	2,480	4,500	3,690	6,000
6	820	1,770	680	2,400
7	1,050	1,310	1,840	1,130
8	650	660	720	480
9	5,760	10,000	7,200	5,600
10	2,070	1,700	2,250	1,300
11	3,410	12,800	12,400	22,100
12	240	1,880	1,870	990
13	540	740	1,530	350
14	130	260	200	140
15	510	160	33	13
16	4,810	10,300	11,500	*
17	190,000	254,000	302,000	*
18	20,600	16,000	16,500	20,100
19	263,000	158,000	412,000	423,000
20	2,490	67,000	36,500	11,600
21	210	3,800	7,800	7,400
22	640	8,400	26,100	24,700

* Counts not done.

Table 5. *The incidence of different organisms on MacConkey's agar with (A) and without (B) pre-incubation at 37° for 2 hr*

Organism and type	Incidence (%) for	
	A	B
Escherichia coli I	53·5	31·9
E. coli II	0·0	12·8
Citrobacter freundii I	2·2	2·1
Cit. freundii II	2·2	0·0
Klebsiella aerogenes I	13·3	10·6
K. aerogenes II	2·2	0·0
K. cloacae	0·0	6·4
Irregular I	2·2	0·0
Irregular II	0·0	4·3
Irregular III	0·0	12·8
Irregular IV	2·2	2·1
Irregular V	4·4	2·1
Not typed	17·8	14·9

The numbers of colonies which developed from the same sample with different pre-incubation periods are shown in Table 4. There was a significant increase in colony count when the inoculated medium was incubated at 37° before incubating at 44·5°. For practical reasons a pre-incubation period of 2 hr is recommended. Another interesting point is that this pre-incubation period not only increased the number of colonies, but also improved the selectivity of the medium for *E. coli* I. The selectivity of the medium, with and without a pre-incubation period, is shown in Table 5.

A comparison was again made between the MPN and the modified roll tube techniques with pre-incubation for 2 hr at 37°. The results (Table 6) show that a colony count close to the MPN can be obtained without a serious increase in the coefficient of variation.

Table 6. *A comparison of the counts obtained by the MPN and roll tube methods after pre-incubation for 2 hr at 37°*

Sample no.		Viable counts* (/ml) by	
		MPN method	Roll tube method
1		78,000	64,600
2		45,000	48,000
3		18,000	46,000
4		20,000	53,300
5		68,000	52,000
6		61,000	44,000
7		130,000	44,600
8		78,000	60,700
	Arithmetic mean	62,250	51,650
	Coefficient of variation	58·11%	14·7%

* Counts done in MacConkey's medium at 44·5°.

Discussion

According to the Ministry of Health (Report, 1957*a*), the MPN technique as applied to the enumeration of *E. coli* I is not intended to estimate the exact number of coli-aerogenes organisms present but the maximum number that may be present. This technique has received some criticism in the past and Swaroop (1956) pointed out that dilution techniques used for enumerating organisms are usually of low precision.

The present investigation of the roll tube technique has indicated that this appears to be more satisfactory for estimating small differences in the concentration of faecal bacteria in water than the MPN technique. Evidence has been obtained which indicates that a pre-incubation period is necessary if maximum counts are to be obtained. It is suggested that this pre-incubation period is effective in reducing the effects due to large thermal differences which would occur when bacteria are suddenly transferred from water sample temperature to 44·5°.

Pre-incubation at 37° possibly allows acclimatization of the bacterial flora. In this respect it is possibly significant, because when pre-incubation was applied to the MPN technique no significant increase in bacterial count was noted (Table 6) over that obtained without pre-incubation (Table 2). The broth medium used for estimating MPN has a low thermal conductivity and the medium only reaches incubation temperature after some time. The greater surface area provided by the roll tube, however, would enable the medium temperature to rise more quickly.

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A NEW TEST FOR THE DETECTION OF BACTERICIDAL ACTIVITY USING AGAR 'REPLICATES'

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SUMMARY: A quick method is described which may be used for testing the bactericidal activity of compounds in solution or in formulation and is particularly useful for examining antiseptic creams. It is based upon making impressions or 'replicates' by agar to agar contact.

The technique has been developed in an attempt to produce a quick and reliable method for detecting bactericidal activity. It is an adaptation of the velvet replica plate technique (Lederberg & Lederberg, 1952). In principle the method consists in obtaining an impression (or 'replicate') on agar from another agar surface upon which a test organism and the preparation under investigation have been in contact. The 'replicate' is then incubated and the area of growth, which is a measure of organisms surviving the contact period, examined. A control 'replicate' is made at the same time in order to detect any bacteriostatic amounts of the active compound which may be carried over.

The main advantages of the technique are that it makes use of readily available materials, for no special apparatus is needed, and that it requires less preparation than the velvet method.

Experimental

Materials. The following materials were used: nutrient broth containing 2·0% of agar (New Zealand, Davis); McCartney or similar shallow bottle tops (not less than 3 cm in diameter), easily sterilized, without liners, possessing lips which enable flat non-convex agar surfaces to be obtained; sterile filter paper (Whatman no. 1) and sterile tissue paper; large square plates (10 in. × 10 in.) for the sown agar and as containers for the agar tops.

Method. Impressions were made by the direct contact of two agar surfaces. One surface consisted of the agar top as described below; the other surface was the one on which the active compound and the test organism were brought in contact, the agar having been seeded with a 1·0% inoculum taken from an overnight broth culture of the test organism.

The bottle tops were filled with unseeded agar so that the agar surface was well up on the lip. To obtain satisfactory results the caps were placed on a perfectly level surface during filling and the agar was used at the lowest temperature possible to avoid condensation and any rapid shrinkage. Any bubbles which formed were eradicated by flaming. Tops prepared in this way are referred to below as 'agar tops'.

When an antiseptic cream was to be examined, sterile tissue paper was placed on the surface of the sown agar to facilitate subsequent removal of the cream. The tissue was smoothed out and excess moisture removed by means of sterile filter paper. Previous experiments had shown that tissue paper was more suitable for this purpose than membrane filters, filter paper (Whatman no. 1) or lawn cotton. Although there has been no difficulty with the samples so far tested, it is advisable to assess the ability of new compounds to diffuse through the paper.

The creams were then placed on the tissue paper and their positions marked on the underside of the plate. The first replicate was made by inverting the unseeded 'agar top' on to a sterile filter paper in order to ensure a dry surface. If the surface was dried by incubation, shrinkage of the agar occurred. Then it was placed on the seeded plate at the original site of the test material, pressed down lightly, removed and incubated as required.

In order to test for 'carry over' of bacteriostatic amounts of the active compound, which could vitiate the test, further impressions were made with seeded or unseeded 'agar tops' either from the site at which the first replicate was made or from a duplicate site. No significant difference has been detected between the amounts of compound carried over by these two procedures. If these replicates were made with a seeded 'agar top' they were ready for incubation; if unseeded, however, the surfaces were streaked with the test organism and incubated. The latter method is advisable if pathogenic organisms are used. However, it is important that the streaking is even and not too heavy or 'carry over' may not be adequately detected. Bactericidal activity is indicated by absence of growth in the centre of the first 'replicate' and complete growth on the control seeded or streaked 'agar top'.

RESULTS

This method has been compared with the velvet pad technique with almost identical results. Antiseptic creams were used in this comparison and some results are shown in Table 1. It can be seen that the replicate method clearly detected bactericidal activity uncomplicated by 'carry over' in creams 1, 2 and 5, whereas creams 7 and 8 showed 'carry over'. Creams 4, 6 and 9 showed no bactericidal activity. The two methods gave similar results with other organisms.

As a further test benzylpenicillin was used for 'carry over' experiments (Table 2). The antibiotic at different concentrations was streaked over the surface of an agar plate and left 2 min before replicates were made with seeded agar or with unseeded agar which was later streaked with culture. After incubation there was little difference between the amounts of penicillin carried over by both methods. In order to determine if the amount of inoculum used would affect this result, 0·5% and 0·25% inocula were employed as opposed to the standard 1·0%, but there was no difference in the results.

Experiments were also carried out to determine the number of cells carried over on the 'agar tops' compared with the number on the velvet. Varying percentages of the inoculum were flooded over agar surfaces and allowed to dry. Sections were cut out and rinsed in broth for 1 min and a count made of each to determine the initial number of cells. Samples of the same size were taken from the replicates and

Table 1. *A comparison of the bactericidal activities of antiseptic creams and other materials as determined by the 'agar top' and velvet replica methods*

Cream no. or bactericide	Results* obtained by			
	'Agar top' method		Velvet method §	
	Growth on replicate†	'Carry over'‡	Growth on replicate†	'Carry over'‡
1	−	−	−	−
2	−	−	−	−
3	±	−	+	−
4	+	−	+	−
5	−	−	−	−
6	+	−	+	−
7	−	+	−	+
8	−	+	−	+
9	+	−	+	−
Dichlorobenzyl alcohol, 2·0%	+	−	+	−
Dichlorobenzyl alcohol, 2·0%; cetrimide, 0·5%	+	−	+	−
Cetrimide, 0·5%	+	−	+	−
Hexachlorophene, 0·5%	+	−	+	−

* The bactericidal activity was determined against *Staph. aureus* and the contact time was 2 hr at 37°.

† Growth on replicate: +, marked; ±, slight; −, absent.

‡ 'Carry over': +, detected; −, not detected.

§ Replica impression made on a seeded plate.

Table 2. *A comparison of the extents of 'carry over' of benzylpenicillin by the 'agar top' and velvet replica methods*

Concn of penicillin (u./ml)	Results* obtained by		
	'Agar top' method		Velvet method‡
	Seeded	Unseeded†	
16	+	+	+
8	+	+	+
4	+	±	±
2	−	−	−
1	−	−	−

* Replicates were made from separate areas of plates containing the antibiotic; 'carry over' (detected with *Staph. aureus*): +, detected; −, not detected; ±, detected to a small extent.

† 'Agar top' subsequently streaked before incubation.

‡ Replica impression made on a seeded plate.

similarly treated, and counts compared. *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used in these tests. The 'agar tops' carried over a greater number of cells; although the velvet initially picked up a similar number, most of these cells did not come off on the replicate but adhered to the surface of the velvet.

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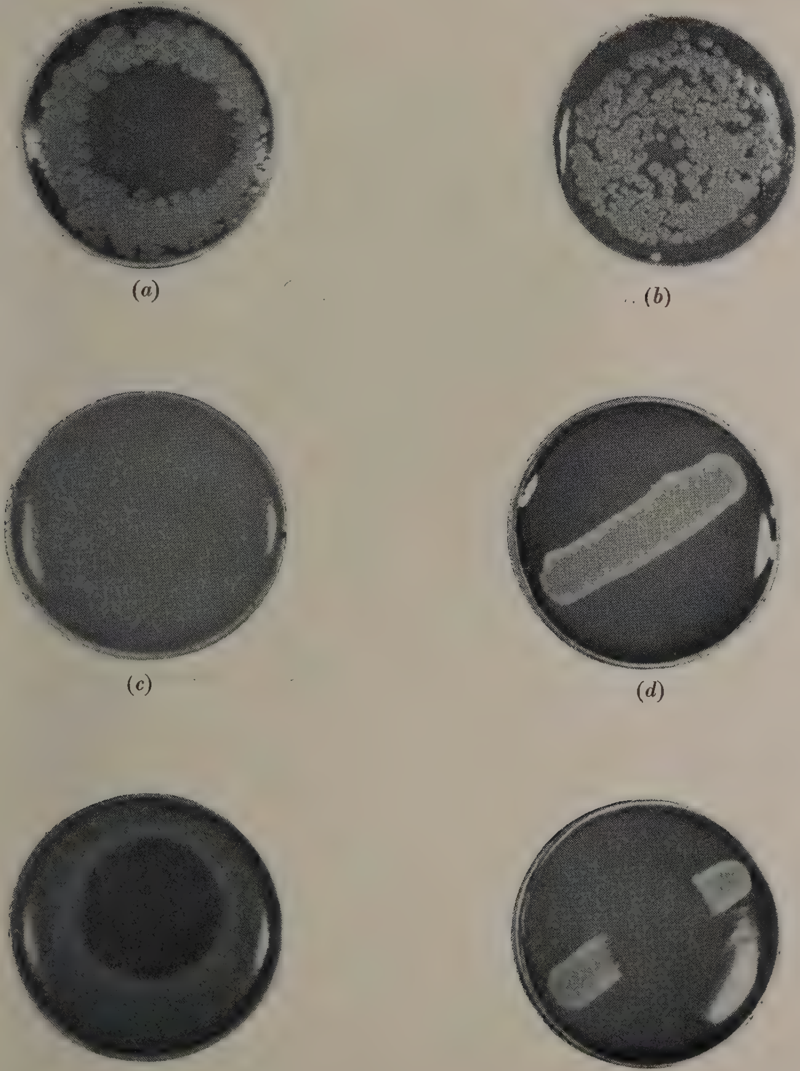

(*a*) (*b*)

(*c*) (*d*)

(*e*) (*f*)

JOY PARRY—A NEW TEST FOR THE DETECTION OF BACTERICIDAL ACTIVITY USING AGAR 'REPLICATES'. PLATE 1

DISCUSSION

The method described above for testing the bactericidal activity of compounds has the advantages of being quick and economical, for it dispenses with the continual preparation of velvet pads and makes use of ordinary laboratory equipment.

It may not be possible to test highly bacteriostatic substances for bactericidal activity by either the 'agar top' or the velvet replica method because of the amount of 'carry over'. However, this can be partially or completely overcome by placing the replicas in a refrigerator before incubation to allow the active compound to be diluted at the surface by diffusion down through the agar before growth commences. Also, in order to reduce the effect of 'carry over', inhibitors can be added to the agar in individual tops; for example, ovolecithin has been used successfully to inactivate the quaternary ammonium compounds.

The technique has been adapted (Metcalf, pers. comm.) to detect bactericidal activity during the routine evaluation of potential antibacterial and antifungal agents. For this purpose the agar tops are replaced by small aluminium caps (Oxoid 12 mm) with $\frac{1}{8}$ in. holes drilled in their bases; these are sterilized, open end downwards, in flat bottomed tins. Melted agar is added until the caps are just submerged, thus producing a perfectly level surface of solidified agar at the open end of each cap. During routine screening, organisms are inoculated on to the surface of nutrient agar containing the compound under investigation by means of a multipoint inoculating device. After overnight growth the bacteriostatic activity of the compound is noted and then replicates are made with the 'agar tops' (prepared as described above) from the small circular areas of inoculation. At the same time a 'carry over' replicate control is made from the uninoculated area of the plate.

The method has proved to be simple in operation and comparisons between it and conventional methods, using a random selection of compounds and several known antibiotics, gave satisfactory results.

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EXPLANATION OF PLATE

PLATE 1. 'Agar top' replicates.

(a) Absence of surface growth in the centre of the 'agar top' indicates bactericidal activity.

(b) Surface growth over the whole area indicates absence of bactericidal activity.

(c) and (d) Absence of 'carry over' of the active compound is shown by complete growth. In (c) the agar was seeded with the test organism before the impression was taken and in (d) the 'agar top' was streaked with the test organism after the impression was made.

(e) and (f) Incomplete growth and areas of inhibition indicate 'carry over'. These correspond to (c) and (d), respectively.

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THE EFFECT OF FEEDING COLICINOGENIC *ESCHERICHIA COLI* ON THE INTESTINAL *E. COLI* OF EARLY WEANED PIGS

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SUMMARY: The *Escherichia coli* contents of samples of faeces from 24 early weaned pigs were estimated qualitatively and quantitatively in two experiments. At weaning (10 days of age) the *E. coli* content was about 10^8/g of faeces. Haemolytic *E. coli*, often pathogenic serotypes, emerged at 12–20 days of age and then tended to disappear at 30–40 days of age. Colicinogenic *E. coli*, isolated from the faeces of pigs from another herd, were grown in a 50 l. batch fermenter and added to the diet of the early weaned pigs, from weaning and until the pigs were 40 days old. Within a few days of feeding the colicinogenic *E. coli*, colicinogenic types formed 20–50% of the coli-aerogenes flora of the faeces. The feeding and establishment of colicinogenic *E. coli* did not suppress the haemolytic *E. coli* and did not affect the incidence of mortality among the pigs.

Previous work established a connection between oedema disease in young pigs and the presence of haemolytic strains of *Escherichia coli* (Lemcke, Bellis & Hirsch, 1957). This agrees with the observations of other workers (Schofield & Davis, 1955; Gregory, 1955; Timoney, 1956; Sojka, Erskine & Lloyd, 1957). Young pigs seemed most susceptible to the disease 7–14 days after weaning (Schofield & Nielson, 1955; Lloyd, in the discussion to the paper by Timoney, 1957).

Lemcke & Hurst (1961) observed that the dominant serotypes in early weaned pigs can suddenly change. The symptoms characteristic of oedema disease may no longer be in evidence but scouring then usually becomes prevalent. Pigs may, therefore, have different dominant serotypes of haemolytic *E. coli* and different disease symptoms, the most obvious of which is scouring.

In attempts to find a practical remedy for scouring the feeding of strains of *E. coli* not usually associated with the disease has been tried. It was hoped that these would displace the strains causing disease. Colicinogenic *E. coli* were used in preference to other strains because it seemed that they would displace the dominant types in the gut more readily; also, the colicinogenic property could be used as a marker to follow the passage of such organisms through the animal.

Colicinogenic strains of *E. coli* produce antibiotics termed colicines; considerable literature has accumulated during the last 60 years on the inhibitory phenomena associated with *E. coli* (see Fredericq, 1948). The antibiotics are readily produced on agar but culturing on cellophane is required for their production in liquid media. Different strains produce various antibiotics all of which are selective and inhibit only Gram-negative organisms, particularly other strains of *E. coli*. Methods for isolating these strains and assaying the antibiotic were reported by Heatley & Florey (1946).

The experimental work is described in two parts: the techniques used for following the disease, and the application of colicinogenic *E. coli*.

MATERIALS AND METHODS

Assessment of disease. Samples of faeces were taken daily whenever possible. About 1 g was accurately weighed into a sterile stainless steel tube containing 6 stainless steel ball bearings of 0·25 in. diam. A volume of quarter-strength Ringer's solution equivalent to 9 times the weight of faeces was added, and the tube vigorously shaken for 2 min. The 10^{-1} dilution thus produced was usually of suitable consistency for pipetting, and further decimal dilutions in quarter-strength Ringer's solution were prepared from it in the normal manner. Dilutions in the range of 10^{-4} to 10^{-8} were plated on penicillin-blood agar using a volumetric loop (Berridge, 1954) and an L-shaped stainless steel spreader. The medium was prepared as follows: a layer of nutrient agar containing benzylpenicillin (Crystapen, Glaxo Ltd.) at a concentration of 0·1 i.u./ml was poured into a Petri dish and allowed to solidify. This was covered with a thin layer of nutrient agar containing 5% of oxalated horse blood (Burroughs Wellcome Ltd.) and 0·1 i.u. of penicillin/ml: when solid the poured plates were dried at 45°.

Isolated colonies of coli-aerogenes organisms were usually produced by this method. The concentration of penicillin was sufficient to suppress the growth of lactobacilli and streptococci, and drying the surface of the medium prevented confluent growth. Zones of haemolysis were more clearly visible in the thin layer of blood agar than in plates containing a thicker, single layer; this made differential counting of haemolytic and nonhaemolytic colonies a simple and rapid process.

Representative haemolytic colonies were picked from the incubated plates and investigated in more detail. Biochemical tests of the coli-aerogenes isolates (Report, 1956) showed them to be invariably *E. coli*. Further characterization was by slide agglutination with specific antisera prepared against strains isolated from the pig herd in previous experiments. The serological tests used are described in detail by Lemcke & Hurst (1961).

Selection of colicinogenic E. coli. A modification of the method of Heatley & Florey (1946) was used. Nutrient agar plates were dried and then inoculated with cultures of *E. coli* isolated from samples of faeces. These samples were obtained from a herd in which antibiotic feeding was not used and which was free from scouring. Two strains of suspected colicine producers were streaked on each plate in such a manner that two straight lines of confluent growth about 2 in. apart were produced. After overnight incubation at 37°, the cultures were killed with chloroform vapour. This was done by placing discs of filter paper soaked in chloroform in the lids of the Petri dishes at room temperature for 30 min; the paper was then removed and the plates incubated with the lids raised for a few hours at 37° to drive off residual chloroform.

The test organisms, haemolytic *E. coli* of serotypes E4 and E57 (Sojka *et al.* 1957), were next streaked across the plates to produce lines of confluent growth at right angles to the killed cultures. After incubation for a further 24 hr, the plates were examined for zones of inhibition adjacent to the streaks of colicinogenic *E. coli*.

The colicine producers which caused the most marked inhibition of both pathogenic serotypes were selected and further tested by a new screening test, providing conditions more akin to those in the gut of the pig. MacConkey's broth, which contains bile salts and has a slightly alkaline pH, was used to simulate two of the features of small intestine contents: 0·5% of trypsin (B.D.H.) and 10% of horse serum were also added. Tubes of this medium were simultaneously inoculated with equal volumes of overnight cultures of two organisms. One was a nonhaemolytic, colicinogenic strain of *E. coli* and the other a serotype E57 haemolytic strain of *E. coli* (at that time the predominant pathogen in the pig herd). After inoculation, the surface of the broth was sealed with a layer of sterile petroleum jelly to create anaerobic conditions, thus further simulating conditions in the intestine.

After incubation for 24 hr at 37°, counts on blood agar were done on the contents of the tubes. The lack of haemolysis round colonies of colicinogenic organisms distinguished them from the haemolytic colonies of the test serotype.

Strain C18, which was used in the feeding experiments, was one of 3 strains of colicinogenic *E. coli* which appeared in pure culture after incubation for 24 hr, having achieved complete dominance over the haemolytic serotype E57.

Detection of colicinogenic E. coli *after feeding*. The penicillin blood agar plates prepared for the assessment of the disease were inoculated on the surface with decimal dilutions of faeces. After differential counting, the haemolytic and nonhaemolytic colonies were killed with chloroform vapour and then sprayed with a thick suspension of colicine sensitive *E. coli*. The plates were incubated for a further 24 hr at 37°, and re-examined. Zones of inhibition were seen round colicinogenic colonies whereas the indicator organisms grew up to the edges of the noncolicinogenic (including haemolytic) colonies.

Feeding trials. Twelve piglets were used in each of two feeding experiments, being allocated to the trial immediately after weaning at 10 or 11 days of age. In each of the experiments two groups of 6 pigs were used, one group to act as control. The groups were balanced with respect to sex, weight and litter.

For use as a feed supplement, *E. coli* C18 was grown in a stirred and aerated 50 l. batch fermenter at 37° for one day. The medium contained 1% of peptone and 1% of glucose and was buffered with Na_2HPO_4 and NaH_2PO_4. The cells were harvested in a Sharples supercentrifuge, from which they were recovered as a paste containing 70% of moisture. This paste was mixed with an equal weight of 0·1% peptone water to produce a creamy slurry. This was incorporated uniformly into an antibiotic free proprietary brand of pig meal; 10% (w/w) of slurry was used in the first experiment and 20% in the second.

RESULTS

At weaning, when the pigs were aged 10–12 days, the faeces generally had a high coli-aerogenes count of about 10^8–10^9/g, but haemolytic *E. coli* were not detected (Fig. 1). A few days after weaning (12–20 days of age) haemolytic *E. coli*, often of serotypes E4 and E57, appeared and sometimes became the major component of the coli-aerogenes flora. If the young pigs survived this critical phase the haemolytic *E. coli* declined and with the method used could no longer be detected when the pigs were 30–40 days old; the total coli-aerogenes population also declined.

Fig. 1 shows the pattern of development of coli-aerogenes flora characteristic of scouring troubles and gut oedema in our herd. The emergence and disappearance of haemolytic *E. coli* as part of the total *E. coli* population was considered to be the most reliable indication of potential disease; successful treatments should alter this pattern.

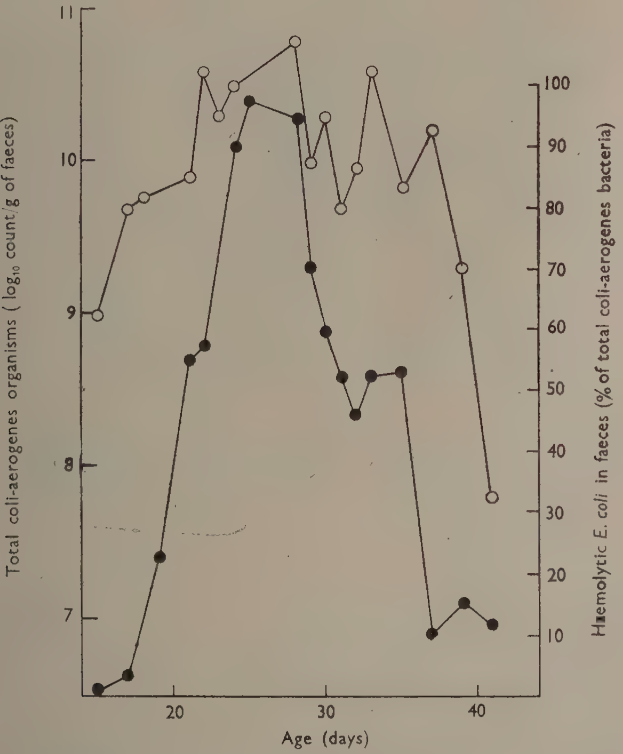

Fig. 1. The total *E. coli* and the percentage of haemolytic *E. coli* in faeces samples from early weaned pigs.
Full circles, haemolytic *E. coli*; open circles, coli-aerogenes bacteria.

In the first experiment in which colicinogenic organisms were given in the diet, the mortality rate was high. Nine of the 12 experimental pigs died, 5 from the control and 4 from the test group and, as only 3 pigs survived, proper bacteriological assessment was impossible. Postmortem examination showed that the deaths were associated with *E. coli* serotype E57 in both control and treated groups. Feeding with colicinogenic strains apparently had no effect, although these organisms were detected in the faeces of every pig of the test group, often in large numbers.

The mortality rate in the second experiment was low and only one pig in the control group died. The results of the bacteriological examination are shown in Fig. 2, in which the percentage of haemolytic *E. coli* in the faeces of the control group is compared with that of the test group. It can be seen that feeding with colicinogenic *E. coli* did not affect the emergence or dominance of haemolytic *E. coli*.

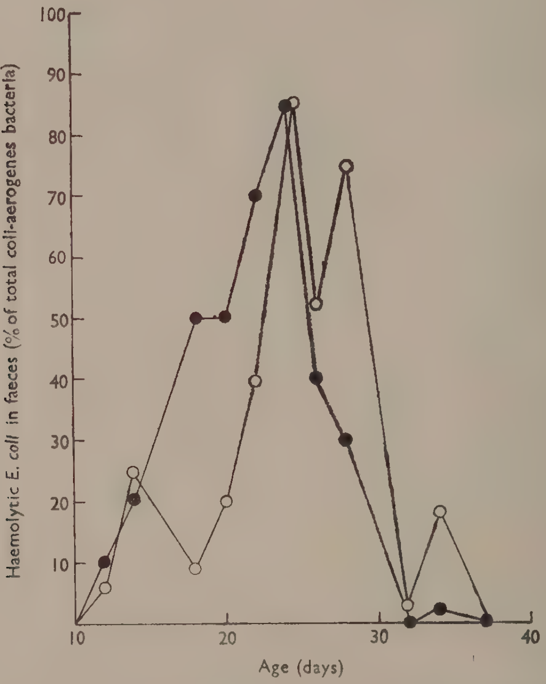

Fig. 2. The emergence of haemolytic *E. coli* in faeces samples from two groups of early weaned pigs.
Full circles, control group; open circles, group fed with colicinogenic *E. coli*.

Fig. 3 gives the results of the examination of samples of faeces obtained from the test group. Colicinogenic strains were established at the same time as the haemolytic *E. coli* emerged and when the pigs were about 20 days old these organisms formed about half of the coli-aerogenes flora of the faeces. In spite of this, however, the haemolytic flora, which consisted mostly of serotypes E4 and E57, predominated and multiplied to give very high numbers. When the pigs were about 30 days old a new factor(s) became operative which depressed both the haemolytic and the colicinogenic populations. The decline of the total coli-aerogenes population at this stage is also shown in Fig. 1.

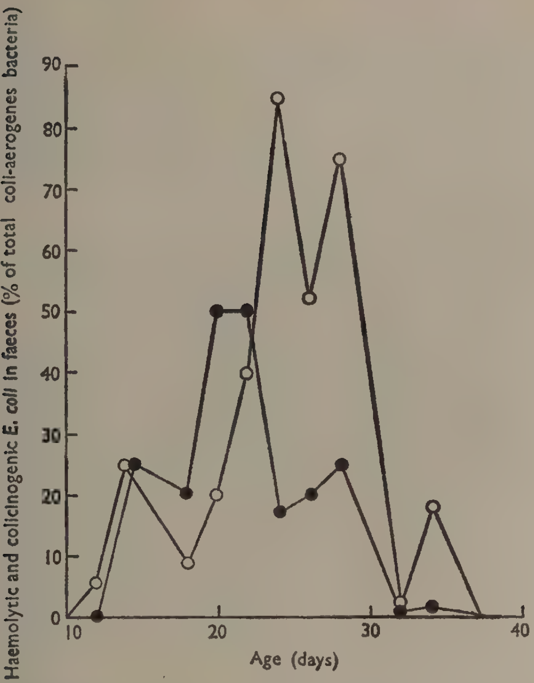

Fig. 3. The emergence of haemolytic and colicinogenic *E. coli* in faeces samples from a group of early weaned pigs fed on a diet containing colicinogenic *E. coli*.
Full circles, colicinogenic *E. coli*; open circles, haemolytic *E. coli*.

DISCUSSION

During the growth of young pigs the dominant strain of *E. coli* in the intestine may become displaced by other strains. For example, Lemcke & Hurst (1961) injected into sows specific vaccines which, it was hoped, would give rise to sufficient antibody in the colostrum to protect young pigs against scouring. Although high antibody titres were obtained, scouring and mortality were not affected, because between the times of vaccination and farrowing the dominant strains changed throughout the herd. Sears, James, Saloum, Brownlee & Lamoreux (1956) observed in man and dogs that, whereas one serotype of *E. coli* always dominated, a second type persisted in small numbers. For no apparent reason the dominant strain would suddenly decline and the strain which hitherto occurred only in small numbers would become dominant. Eventually this process would be repeated and a new dominant strain would emerge. These workers fed dogs with cultures of known types of *E. coli* but were unable to change this pattern. An understanding of the causes of the dominance and later disappearance of a given strain would be of help in attempts to control scouring conditions in animals.

It is possible that antagonistic relationships among the flora in the intestine could account for these phenomena, and it was therefore logical to test the colicines produced by *E. coli* previously isolated from the intestine. Two experiments in which these organisms were fed and established in the pigs showed that the dominant haemolytic strains could not be suppressed by this means.

Without a programme of slaughter, it is impossible to say how many of the colicinogenic organisms became established in different parts of the intestine. This is rather important because, although they appeared in the faeces, the numbers of colicinogenic organisms could have remained low in the small intestine, which may well be the site of multiplication of haemolytic *E. coli*. It would be necessary to carry out slaughtering experiments before it could definitely be concluded that colicines are not one of the factors responsible for the successive dominance of strains.

This possibility is strengthened by the discrepancy between the *in vitro* and *in vivo* results. Feeding and establishment of colicinogenic *E. coli* did not affect the dominance of haemolytic *E. coli* strain E57. *In vitro*, however, under anaerobic conditions and in the presence of trypsin and bile salts, colicinogenic *E. coli* C18 became completely dominant over haemolytic strain E57 in mixed culture incubated overnight. Since these conditions resemble those *in vivo* it may be that the organisms contained in the diet do not become established in the correct portion of the gut, or do not produce colicine.

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THE BACTERIOLOGY OF ICE CREAM STORED IN DOMESTIC REFRIGERATORS

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SUMMARY: Retail blocks of ice cream from three manufacturers were stored for 4 weeks in domestic refrigerators set at 'normal' and 'lowest' temperatures. Samples from the surface of each block were taken at intervals of a week for one month and were tested by colony counts at 30° and at refrigerator temperature. Samples of the bulk of each block were examined by colony counts and methylene blue reduction tests. There were few bacteria in the standard ice cream and these did not increase in number during the storage period. The dye reduction time of one product was slightly shorter after the sample had been stored; the rest remained unchanged. The reduction time of the sample containing strawberry flavouring and colouring was shorter than that of the corresponding vanilla ice cream and decreased more rapidly. 'Dairy' ice cream contained more bacteria than the standard types and had a very much shorter initial reduction time. The only effect of storage of this ice cream was a slight reduction in bacterial numbers. Storage for a month induced surface hardening which made the ice cream unpalatable, but there was no bacterial increase and it was concluded that domestic storage of ice cream does not increase the bacterial load.

Although ice cream produced commercially in Britain to-day is generally of high bacteriological quality, it was thought to be of interest to determine the effects of storage under various domestic conditions on the counts of mesophiles and psychrophiles and on the methylene blue reduction times of a variety of products.

Materials and Methods

Equipment used. Two refrigerators of 3·6 cubic feet capacity and two of 5 cubic feet were used. These were obtained from the production line by courtesy of one manufacturer. The freezing units of these maintained temperatures over the ranges −5° to −13° ('coldest' settings) and −4° to −10° ('normal' settings). In these were stored ice cream blocks purchased from retail shops and transported to the laboratory in vacuum flasks.

Products examined. The ice creams included the 'standard' vanilla product of three manufacturers (WL/S, MC/S and LL/S), a 'standard' strawberry-vanilla product of one manufacturer (LL/SV) and a 'dairy' vanilla product of one manufacturer (MC/D). This ice cream (MC/D) was stored in the normal way and as superimposed packs at both normal and coldest settings.

Sampling. Each refrigerator contained two blocks of each of the products examined, one of which was opened normally and left loosely wrapped (open packs). The other was opened aseptically at one end, a sample removed, and the cardboard refolded to be in contact with the residual ice cream (closed packs). The samples were of two kinds; the surface layer to a depth of about 2 mm was removed with an alcohol-sterilized scalpel, after which a complete cross section of the block was removed. Samples had to be removed with an alcohol-sterilized spatula from ice cream packs which had been stored on top of other packs because the product was semiliquid.

Bacteriological examination. Both types of sample were tested by a standard plate colony count on Yeastrel milk agar incubated for 2 days at 30°. The 'dairy' ice cream count was repeated at 3 and 5 days. The surface samples were also plated and incubated for 6 weeks in the cabinet space of the refrigerators (0–5°) to estimate the numbers of psychrophiles, and the bulk samples were subjected to methylene blue (M.B.) reduction tests after one-fifth dilution in quarter-strength Ringer's solution and ageing for 17–20 hr at 20°.

RESULTS

There was no appreciable difference between the colony counts obtained from the surface samples and those from the cross section bulk samples. Occasional higher counts in the surface samples probably indicate contamination during sampling. The 'standard' products had low initial counts which did not appreciably alter during the four weeks of storage (Tables 1 and 2). There was no increase in the psychrophilic count for two of the manufacturers' samples; the third (MC/S) showed an increase in fungal count (from <10/ml to about 200/ml) over the period of storage (Table 3). This particular product was also the only 'standard' ice cream to show a decrease in the M.B. reduction time, the major change occurring in the fourth week of storage (Table 4). The M.B. reduction time also appeared to be shortened by the strawberry

Table 1. *The effect of storage on the numbers of bacteria in two standard vanilla ice creams (WL/S and MC/S)*

Type of sample	Condition of packs	Refrigerator capacity (ft^3) and setting*	Colony count in indicated products after storage for (days)											
			WL/S						MC/S					
			0	3	7	14	21	28	0	2	7	14	21	28
Bulk	Open	5 A	<100	10	10	10	40	10	20	20	<10	20	10	110
		5 B	<100	40	20	10	10	<10	50	10	10	<10	30	100
		3·6 A	<100	<10	30	<10	10	<10	40	50	10	<10	20	10
		3·6 B	<100	40	<10	10	30	<40	30	50	20	30	30	40
	Closed	5 A	200	30	20	10	20	10	20	20	10	20	10	20
		5 B	<100	30	30	20	40	<10	50	60	10	50	10	70
		3·6 A	<100	10	120	<10	20	<10	50	20	20	40	60	30
		3·6 B	<100	10	10	20	30	<10	30	20	20	20	30	40
Surface	Open	5 A	<100	40	20	<10	30	10	60	20	10	10	80	100
		5 B	<100	30	70	20	30	13×10^4	50	30	50	20	80	60
		3·6 A	100	16×10^3	<10	4,500	20	10	60	30	10	20	20	90
		3·6 B	<100	390	40	20	<10	30	40	860	40	20	40	40
	Closed	5 A	<100	110	20	570	30	20	5×10^6†	30	20	60	250	140
		5 B	200	50	150	10	10	640	20	10	20	30	40	20
		3·6 A	<100	60	10	30	20	20	80	40	10	10	60	60
		3·6 B	100	70	2,260	60	20	20	30	80	10	80	40	60

* Refrigerator settings: A, 'normal'; B, 'coldest'.

† This figure is suspect because it followed experiments in which contaminated pipettes and media were discovered.

Table 2. *The effect of storage on the numbers of bacteria in one standard vanilla (LL/S) and one strawberry-vanilla (LL/SV) ice cream*

Type of sample	Condition of packs	Refrigerator capacity (ft³) and setting*	Colony count (/ml) in indicated products after storage for (days)									
			LL/S†					LL/SV†				
			0	7	14	21	28	0	7	14	21	28
Bulk	Open	5 A	60	270	50	30	30	—	—	—	—	—
		5 B	—	—	—	—	—	210	60	40	90	20 30
		3·6 A	70	180	20	30	10	—	—	—	—	—
		3·6 B	—	—	—	—	—	30	60	20	50	10 40
	Closed	5 A	60	110	10	90	30	—	—	—	—	—
		5 B	—	—	—	—	—	40	70	20	40	10 20
		3·6 A	1,880	110	10	80	10	—	—	—	—	—
		3·6 B	—	—	—	—	—	120	90	60	80	20 30
Surface	Open	5 A	40	400	30	30	40	—	—	—	—	—
		5 B	—	—	—	—	—	2,700	300	40	50	200
		3·6 A	20	30	<10	20	120	—	—	—	—	—
		3·6 B	—	—	—	—	—	40	60	10	40	130
	Closed	5 A	30	40	30	40	40	—	—	—	—	—
		5 B	—	—	—	—	—	550	40	30	30	50
		3·6 A	150	80	30	50	60	—	—	—	—	—
		3·6 B	—	—	—	—	—	190	240	40	30	160

* Refrigerator settings: A, 'normal'; B, 'coldest'.

† The standard vanilla product of this manufacturer was stored in each case at the 'normal' settings of the refrigerators and the strawberry-vanilla product at the 'coldest' settings; where two results are given on a single occasion the upper one refers to the strawberry flavoured section and the lower one to the vanilla flavoured section of a mixed block.

Table 3. *The effect of storage on the numbers of psychrophiles in ice cream*

Condition of packs	Refrigerator capacity (ft³) and setting*	Colony counts (/ml) in indicated products† after storage for (days)										
		WL/S					MC/S		LL/S		LL/SV	
		0	7	14	21	28	0	28	0	28	0	28
Open	5 A	<10	<10	<10	<10	<10	<10	20	40	<10	—	—
	5 B	<10	<10	<10	<10	<10	<10	260	—	—	1,100	10
	3·6 A	<10	<10	<10	<10	<10	<10	210	20	10	—	—
	3·6 B	<10	10	<10	10	<10	<10	60	—	—	50	<10
Closed	5 A	<10	<10	<10	<10	<10	<10	400	10	<10	—	—
	5 B	<10	<10	<10	20	10	20	240	—	—	40	<10
	3·6 A	10	10	<10	<10	<10	<10	150	70	<10	—	—
	3·6 B	<10	<10	10	10	<10	<10	10	—	—	100	<10

* Refrigerator settings: A, 'normal'; B, 'coldest'.

† Standard vanilla products: WL/S, MC/S and LL/S; strawberry-vanilla product: LL/SV.

ingredients in the strawberry-vanilla (LL/SV) ice cream. At the beginning of the experiment the complete samples (LL/SV) (i.e. those containing the two flavours mixed together) had a reduction time 2 hr shorter than that of the vanilla ice cream (LL/S). After 4 weeks storage a difference of ½ hr was found between the reduction times of the strawberry and the vanilla components of the same block (Table 4).

The 'dairy' ice cream started with a greater number of bacteria than the 'standard' ice cream, but these numbers tended to decrease on storage even when the temperature was raised (superimposed packs at a 'normal' setting) (Table 5). The M.B. reduction times were low, but not consistently so.

Table 4. *The effect of storage on the methylene blue reduction times of ice cream*

Condition of packs	Refrigerator capacity (ft³) and setting*		Reduction time (hr) of indicated products† after storage for (days)											
			WL/S						MC/S					
			0	3	7	14	21	28	0	2	7	14	21	28
Open	5	A	>1½	>4	>6	>6	>5	>5½	4	4½	3	4	4½	2
		B	>1½	>4	>6	>7	>5	>5½	5½	4½	4	<7	4	2
	3·6	A	>1½	>4	>6	>7	>5	>5½	4	4	<5	3	3½	3½
		B	>1½	>4	>6	>7	>5	>5½	4	4	4	4	4½	2
Closed	5	A	>1½	>4	>6	>7	5	>5½	5½	4	3	7	3	3
		B	>1½	>4	5½	>7	5	>5½	5½	4½	4	3½	3½	3½
	3·6	A	>1½	>4	>6	>7	>5	>5½	4½	4½	5½	3	3½	3½
		B	>1½	>4	5½	5½	5	>5½	4½	4	3	4	3	3½

Condition of packs	Refrigerator capacity (ft³) and setting*		LL/S‡					LL/SV‡				
			0	7	14	21	18	0	7	14	21	28
Open	5	A	5½	> 3½	5½	5½	4	—	—	—	—	—
		B	—	—	—	—	—	3½	>3½	4	1½	3 3½
	3·6	A	5½	>3½	5½	3½	5½	—	—	—	—	—
		B	—	—	—	—	—	3½	>3½	4½	1½	3½ 3½
Closed	5	A	3½	>3½	5½	3½	6	—	—	—	—	—
		B	—	—	—	—	—	3½	>3½	3½	2	3½ 3
	3·6	A	3½	>3½	5½	3½	6	—	—	—		
		B	—	—	—	—	—	3	>3½	4½	1½	3½ 4

* Refrigerator settings: A, 'normal'; B, 'coldest'.

† Standard vanilla products: WL/S, MC/S and LL/S; strawberry-vanilla product: LL/SV.

‡ The standard vanilla product of this manufacturer was stored in each case at the 'normal' settings of the refrigerators and the strawberry-vanilla product at the 'coldest' settings; where two results are given on a single occasion the upper one refers to the strawberry flavoured section and the lower one to the vanilla flavoured section of a mixed block.

Table 5. *The effect of storage under different conditions on the colony counts of mesophiles* (*a*) *and psychrophiles* (b) *and on the methylene blue reduction times* (c) *of 'dairy' ice cream*

Position of packs in refrigerator	Refrigerator capacity (ft³) and setting*		Colony counts (/ml) and reduction times (hr) of ice cream stored for (days)										
			0		7		14		21		28		
			(a)	(c)	(a)	(c)	(a)	(c)	(a)	(c)	(a)	(b)	(c)
On the bottom of the freezer	5	A	2000	<2	720	1	70	5	500	2	1,200	170	2
		B	1,050	<2	1,010	1	120	4	360	2	890	70	1½
	3·6	A	1,750	2½	870	1	130	3½	510	1½	270	70	2
		B	1,730	2½	1,020	1	180	3½	660	1½	330	150	2
On the top of other packs	5	A	1,120	<⅓	500	4½	930	1	540	1½	400	30	<½
		B	830	<⅓	600	3	370	½	660	½	540	10	<½
	3·6	A	700	<⅓	340	3	430	1½	390	1½	430	30	0
		B	620	<⅓	60	3	640	1	780	½	800	50	<½

* Refrigerator settings: A, 'normal'; B, 'coldest'.

DISCUSSION

The results showed that all the ice creams tested were bacteriologically acceptable after prolonged storage. The only increases in numbers observed were due to fungi, which may have had an effect on the M.B. reduction time. An attempt to follow changes in the surface flora by a direct (microscopic) count was unsuccessful because there were too few organisms present. The standard plate colony count gave better results, but there were irregular fluctuations, some of which were probably due to contamination during the sampling of the surface ice cream. The remainder of the bacteria did not seem to be located on the surface of the block so that a complete cross section sample was both easier to obtain and more reliable. The methylene blue reduction times also showed variations. Some of these were irregular, some probably due to an increase of fungi during storage and ageing, and some to the physical or chemical effect of the colouring and flavouring in the strawberry ice cream. Because of the effect of such additions the colony count is preferable for assessing the quality of sophisticated ice creams.

Although the bacteriological tests showed the samples to be satisfactory after storage, other deterioration became apparent. Superimposed packs were semiliquid, whereas all other samples developed a tenacious and sometimes granular skin, associated with a tallowy taste. This was more marked in areas in close contact with cardboard and in the closed packs rather than the open packs.

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(Symposium on Bacteria of the Intestine: Paper I)

THE DEVELOPMENT OF THE BACTERIAL FLORA OF THE FAECES OF ANIMALS AND MAN: THE CHANGES THAT OCCUR DURING AGEING

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ALTHOUGH considerable knowledge of the bacterial flora of the faeces of adult animals exists, only a small number of observations dealing with its development in the young domestic mammal have been published. These have been concerned with only a few of the types of bacteria that inhabit the alimentary tract. Recently a more comprehensive study has been performed (Smith & Crabb, 1961), this study being preliminary to the investigation of the bacteriology of diarrhoea in young farm animals. Bacteria such as lactobacilli, bacteroides and streptococci, which are not considered to play an important part in the causation of diarrhoea, were classified on a generic basis with some differentiation according to colonial morphology. Bacteria such as *Escherichia coli* and *Clostridium welchii*, which are sometimes considered to be associated with intestinal disease and/or the growth stimulus obtained by antibiotic

Table 1. *The cultural methods used and the principal bacteria enumerated*

Medium	Atmospheric environment	Incubation time (days)	Bacteria usually enumerated
MacConkey's agar	Air	1	*E. coli* Lactose non-fermenters
Neomycin-Nagler agar[1]	H_2+CO_2	1	*Cl. welchii*
TITG agar[2]	Air	1	'Faecal' streptococci
8% Salt agar	Air	2	*Staph. aureus*
Rogosa's agar[3]	$H_2+5\%\ CO_2$	2	Most lactobacilli
RCM agar[4] +5% blood	$H_2+5\%\ CO_2$	2	'Total' count Some lactobacilli
RCM agar[4] +5% blood +neomycin*	$H_2+5\%\ CO_2$	2	Bacteroides, anaerobic Gram—ve cocci, 'total' streptococci, sporeformers, yeasts, 'actino' types

* Included principally to inhibit *E. coli* and most lactobacilli.

[1], Lowbury & Lilley, 1955; [2], Barnes, 1956; [3], Rogosa, Mitchell & Wiseman (1951); [4], Oxoid.

feeding, were classified according to species. The counting technique employed was based on that of Miles & Misra (1938). The cultural methods and the main types of bacteria enumerated are shown in Table 1, all incubation being carried out at 37°. Counts were carried out daily for the first 2–4 weeks of life, then twice weekly, weekly, fortnightly and finally monthly, depending on the length of the period of observation.

Because of the great similarity between the results obtained for all animals of the same species irrespective of environment, it is possible to illustrate those for the five main types of bacteria found in the faeces of calves, lambs, piglets, human babies and a rabbit in severely diagrammatic form without making any great sacrifice of accuracy (Fig. 1); the full details are given in the published paper. Apart from the main types, anaerobic Gram-negative cocci were occasionally found in the faeces of the calves, lambs, piglets and babies in numbers as high as 10^8/g. Sporeforming organisms and paracolon bacilli were also occasionally present, but in lower numbers, and yeasts sometimes formed a substantial portion of the faecal flora of older calves.

Staphylococcus aureus was found in the faeces of the baby (Fig. 1d) on 24 of 58 occasions. It was never found in the faeces of the other animals.

In general, the development and subsequent composition of the bacterial flora of the faeces of all the animals examined, irrespective of species, were closely similar in early life: but, as they aged, considerable dissimilarities became progressively more apparent. Some types of bacteria became predominant in some species but almost completely disappeared from the faeces of others. This was particularly noticeable in the case of the bacteroides, although it has to be pointed out that the medium on which they were counted, neomycin-blood agar, is not highly selective for these bacteria and they may sometimes have been recorded as being absent from a specimen whereas in fact their presence was masked by other neomycin resistant bacteria. Another dissimilarity between species associated with ageing was that of total bacterial count. For example, the faeces of calves when 6 months or so of age frequently contained 10,000 times fewer viable bacteria than they did when the calves were only a few weeks old. By contrast, no decrease was noted with ageing in the human baby.

Two well defined colonial types of streptococci on thallous acetate-tetrazolium-glucose (TlTG) agar (Barnes, 1956) exhibited a similar characteristic pattern of development in the faeces of the calves, lambs and piglets. The results for one of the calves, a typical example, is illustrated in Fig. 2. These two colonial types were referred to as 'red centred' (*Streptococcus faecalis* or *liquefaciens*) and 'large white' (*Strep. faecium*). This common pattern was not noted in the babies or the rabbit. In one baby examined for a long period of time, 'red centred' streptococci appeared in very early life in large numbers (10^8–10^9/g) and persisted for 7 weeks or so. They were not found at all for the next 24 weeks but then they reappeared and persisted in numbers of 10^5–10^6/g of faeces. 'Large white' streptococci were found only very occasionally during early life in the babies, but they were isolated regularly in small numbers after 4 months of age. 'Large white' streptococci were never found in the faeces of the rabbit and 'red centred' ones only infrequently. The commonest types in human and rabbit faeces grew as small pink colonies on TlTG agar.

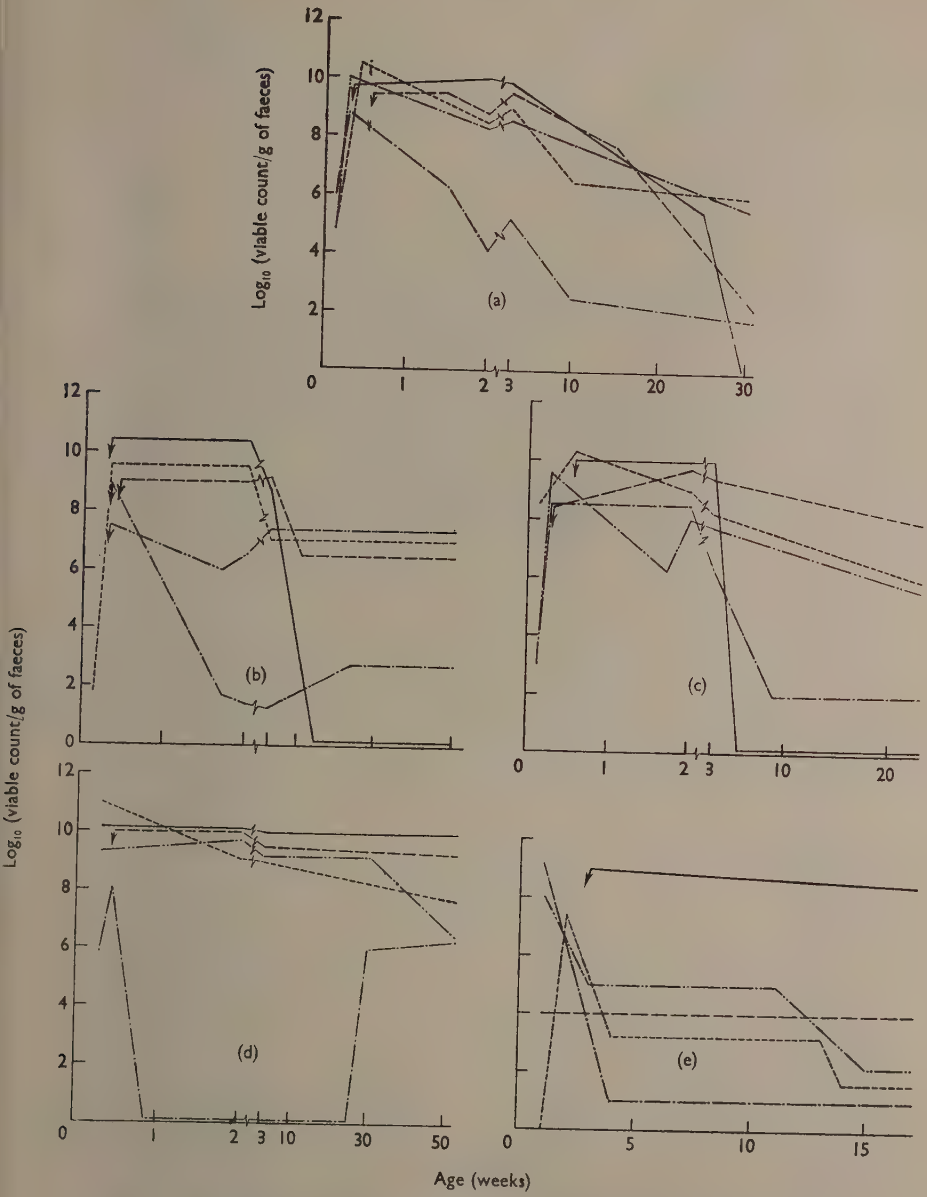

Fig 1.. The influence of age of the animal on the bacterial content of the faeces of a calf (a), lamb (b), piglet (c), human baby (d) and rabbit (e). Entire lines, bacteroides; chain dots, *Clostridium welchii*; short dashes, *Escherichia coli*; long dashes, lactobacilli; double chain dots, streptococci. (From Smith & Crabb, 1961).

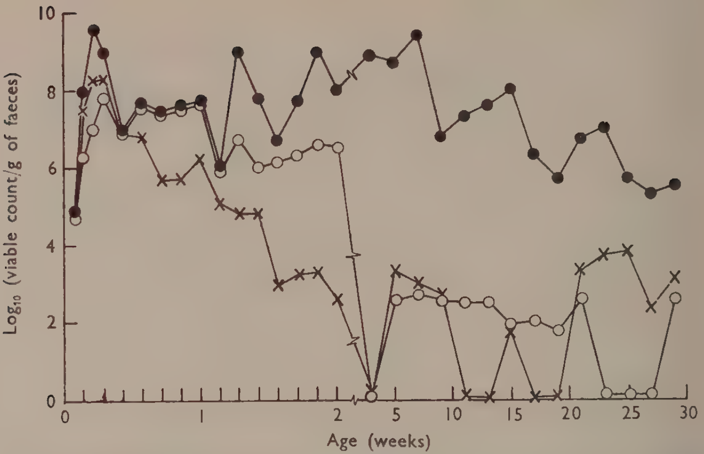

Fig. 2. The influence of the age of a calf on the faecal content of different types of streptococci. Full circles, total streptococci; crosses, streptococci forming red centred colonies; open circles, streptococci forming large white colonies. (From Smith & Crabb, 1961).

Although *E. coli* behaved in a very orderly fashion throughout the examination period of most of the animals, reaching a high peak rapidly and then declining slowly and gradually with age, it is apparent from phage typing studies (Smith & Crabb, 1956) that considerable fluctuations in the numbers of the strains forming the total *E. coli* population may occur, despite the apparent pacific state of this population as a whole. Tables 2 and 3 illustrate this point.

Table 2. *The phage types of* Escherichia coli *present in the faeces of healthy calves*

(From Smith & Crabb, 1956)

Calf no.	Phage types* of *E. coli* in faeces of calves aged (days)											
	1	2	3	4	5	6	7	8	9	10	11	12
1	2 (10)	2 (10)	2 (10)	2 (9) 59 (1)	2 (8) 4 (1) 60 (1)	2 (10)	38 (10)	38 (6) 4 (3) 3 (1)	3 (9) 4 (1)	3 (10)	77 (6) 3 (4)	77 (7) 3 (3)
2	64 (4) 65 (4) 38 (1) 3 (1)	65 (4) 4 (3) 38 (2) 69 (1)	2 (6) 36 (2) 69 (2)	74 (9) 88 (1)	74 (8) 3 (2)	3 (7) 74 (3)	77 (6) 3 (2) 74 (2)	3 (10)	3 (8) 74 (2)	3 (6) 77 (4)	3 (10)	77 (10)
3	71 (5) 36 (5)	2 (3) 3 (3) 71 (2) 101 (2)	2 (7) 62 (2) 71 (1)	71 (6) 2 (3) 3 (1)	36 (4) 22 (2) 83 (2) 63 (1) 1 (1)	71 (9) 1 (1)	63 (6) 71 (2) 64 (2)	63 (4) 2 (4) 71 (2)	2 (8) 71 (2)	2 (4) 71 (4) 63 (1) 60 (1)	2 (6) 71 (2) 80 (2)	2 (4) 71 (3) 80 (2) 88 (1)
4	83 (7) 88 (3)	88 (7) 30 (3)	88 (6) 83 (2) UT (2)	45 (4) 88 (3) 39 (3)	45 (6) 88 (2) 83 (1) UT (1)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)
5	101 (5) UT (3) 72 (2)	101 (5) UT (5)	UT (10)	UT (10)	UT (10)	UT (10)	UT (6) 4 (4)	UT (6) 4 (4)	UT (8) 4 (2)	4 (10)	4 (10)	4 (10)

* Ten cultures were isolated from each sample of faeces and the number belonging to each phage type is given in brackets; UT, untypable.

Table 3. *The phage types of* Escherichia coli *present in the faeces of 3 cows at 6 consecutive weekly examinations*

Cow no.	Phage types* of *E. coli* after (weeks)					
	1	2	3	4	5	6
1	80 (2) 101 (1) UT (1)	101 (3) 82 (1)	101 (2) 45 (2)	101 (4)	101 (4)	101 (3) 3 (1)
2	31 (4)	142 (4)	142 (4)	142 (3) 45 (1)	142 (3) 8 (1)	8 (4)
3	UT (4)	87 (4)	24 (4)	105 (4)	80 (4)	8 (4)

* Four cultures were isolated on each occasion and the number of each phage type present is given in brackets; UT, untypable.

Table 4 summarizes the results (Smith & Crabb, 1961) of the examination of the faeces of 110 fully adult animals, 10 from each of 11 different species, all animals being maintained in different environments. The main points of interest observed in these studies included: the common pattern of the composition of the faecal flora of animals of the same species, and the considerable differences existing between species; the enormous variation in total viable count between different animal species; the low numbers of *E. coli* in guinea pig, rabbit, horse and cattle faeces; the low numbers of *Cl. welchii* in rabbit, guinea pig, mouse and horse faeces

Table 4. *The numbers of viable bacteria found in the faeces of adult animals of different species*

(From Smith & Crabb, 1961)

Species of animal	Median values and ranges of $\log_{10}$ (counts/g of faeces) for				
	E. coli	*Cl. welchii*	Streptococci	Bacteroides	Lactobacilli
Cattle	4·2 (1·7–5·8)	2·5 (0 –3·3)	5·5 (4·3–6·4)	0 (0)	4·0 (0 –5·6)
Sheep	6·3 (5·2–8·0)	3·4 (1·7–5·9)	6·2 (5·0–7·5)	0 (0 –6·4)	5·2 (0 –5·4)
Horses	3·8 (3·0–5·4)	0 (0 –2·3)	7·0 (3·7–8·3)	0 (0 –7·4)	7·0 (5·7–8·0)
Pigs	6·7 (5·2–7·6)	3·7 (2·8–5·7)	6·5 (5·7–8·2)	7·4 (0 –8·0)	8·6 (6·0–9·2)
Rabbits	3·2 (0 –6·7)	0 (0)	4·4 (0 –5·6)	8·7 (7·3–9·1)	0 (0 –6·4)
Guinea pigs	0 (0 –2·7)	0 (0 –2·3)	0 (0 –8·7)	0 (0)	8·3 (7·4–9·3)
Mice	7·1 (4·8–8·9)	0 (0 –2·6)	8·0 (6·4–8·9)	9·0 (0 –10·3)	9·1 (7·8–10·9)
Human beings	7·0 (5·7–8·5)	3·4 (0 –5·9)	6·3 (3·4–9·4)	9·6 (8·3–10·1)	8·8 (7·3–10·0)
Dogs	7·7 (6·2–8·9)	8·4 (4·7–9·0)	7·4 (5·7–10·1)	8·7 (6·5–9·6)	6·0 (0 –9·0)
Cats	7·4 (5·2–9·0)	7·6 (5·5–9·0)	8·3 (4·1–8·9)	9·0 (8·0–10·0)	8·7 (4·3–10·0)
Chickens	6·4 (4·0–7·6)	2·4 (0 –4·4)	7·8 (0 –8·7)	0 (0 –9·0)	8·7 (6·5–9·1)

* The figures represent the approximate numbers commonly found during the examination of 10 animals of each species.

and the high ones in dog and cat faeces; the great variation between the animal species in regard to the numbers of lactobacilli and bacteroides in their faeces; *Staph. aureus* was found in only 3 of the 110 faecal specimens, all 3 originating from human beings.

Although it is not difficult to demonstrate the similarity between the faecal flora of all the very young animals examined, irrespective of species, and the dissimilarity that occurs between species as the animals age, it is much more difficult to produce evidence indicating why this should be so. It may be related to the fact that all mammals when young have the same diet (milk) and a similar alimentary tract, but that as they age their diets become increasingly dissimilar and their alimentary tracts diversely specialized in structure and function. There is considerable published evidence indicating that diet has an important influence on the alimentary flora. However, in a small number of experiments conducted in the present studies it has not been possible to bring about profound changes in the faecal flora by altering the composition of the diet. For example, Fig. 3 illustrates the effect on the *Cl. welchii* content of the faeces of a dog of alternating diets of meat and cereal. Although the change to the cereal diet was usually accompanied by a decrease in the *Cl. welchii* count, the numbers persisting were still higher than those found in most other species of animals.

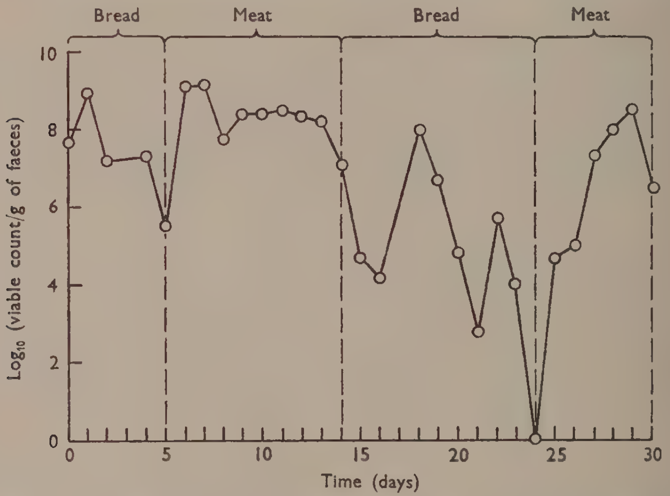

Fig. 3. The effect of alternate all meat and all bread diets on the *Clostridium welchii* content of the faeces of a dog. (From Smith & Crabb, 1961).

Changes much greater than those obtained by altering the composition of the diet were noted in a pig induced to consume large quantities of food by enforced periods of fasting. The main effects were on the *E. coli*, *Cl. welchii* and 'large white' streptococcus contents of the faeces, considerable increases in numbers of these bacteria following closely on the increased consumption of food (Fig. 4).

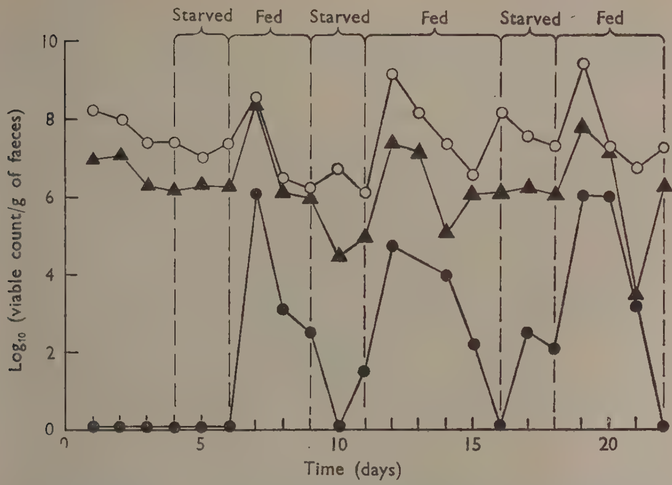

Fig. 4. The effect of alternate feeding and starving on the bacterial flora of the faeces of a dog. Open circles, *Escherichia coli*; full circles, *Clostridium welchii*; triangles, streptococci forming large white colonies. (From Smith & Crabb, 1961).

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(Symposium on Bacteria of the Intestine: Paper II)

SOME RULES IN THE ECOLOGY OF THE INTESTINAL MICROFLORA OF MAN

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1. Introduction

The macro-organism and the micro-organisms which live on its skin and mucous membranes have to be considered as an inseparable ecological system. It is well known from the results of germ free research that the metabolic links between macro-organism and body flora are numerous and important, and may concern many metabolic reactions of the host. The microbial metabolism—mainly that of the intestinal flora—may influence the metabolism of cancers, serum proteins, cholesterol, hormones and vitamins, and affect the incidence of caries and the functioning of the body defence mechanism, to cite only some important examples. Experiments with the feeding of antibiotics to farm animals under practical conditions indicate the probability that under normal conditions the net effect of the body flora is detrimental; thus the protection of the animal against undesirable microbial processes or metabolites may be inadequate to afford optimal thriving. It is possible that a similar state of affairs exists in man, at least in certain circumstances, for instance in children with disease or malnutrition, or after premature birth.

Although there is no doubt about the importance of the body flora to the host, our knowledge, even of simple facts, is surprisingly small. The data concerning the composition of any natural microflora, and the criteria upon which the terms 'normal' or 'abnormal' microflora are based, are insufficient and poor. Some examples may illustrate that common opinions are dubious or wrong. In breast fed babies the intestinal flora is said to be composed almost exclusively of *Lactobacillus bifidus*, which suppresses all other organisms, especially the coli-aerogenes group. At weaning

L. bifidus is said to disappear because only mother's milk contains a factor (the so-called bifidus factor) which enhances and protects the organism. As a result of a diet of cow's milk the coli-aerogenes bacteria are said to become the most numerous, all other organisms being only a small minority. It will be shown later that our results are at variance with this. The idea that the dominant flora, even in adults, is composed of coli-aerogenes bacteria has led to the theory of the so-called 'coli-dysbactery' (Baumgärtel, 1954; Nissle, 1956). According to these authors lactose splitting coli-aerogenes organisms are normal, and are beneficial to the host and a symptom of wellbeing; lactose negative types, however, are said to be degenerate and at least a symptom of disease, if not the cause of it. There exists an enormous volume of literature, mostly in Germany, concerning the elimination of these 'bad' coli-aerogenes bacteria by the ingestion of living cultures of 'good' ones.

The exaggerated importance attached to the coli-aerogenes bacteria resulted from false suppositions by Kohlbrugge (1901) who, with great authority, cast doubt on the existence of any anaerobic bacteria in the intestinal canal of the adult. Other misconceptions arose from the belief that the intestinal flora is indispensable for man and higher animals. It is further questionable whether intestinal organisms supply the host with vitamins in any appreciable amount. It is the opinion of the author that the intestinal microflora has no general important benefit.

2. AIMS

It was the purpose of this work to determine qualitatively and quantitatively the composition of the cultivable part of a natural microflora.

The term 'microbiocenosis' has been adopted to characterize the typical microflora of an organ or part of an organ. This is based on the term 'biocenosis' used in macro-ecology. The situation in a normal, balanced, reproducibly composed, naturally occurring microbiocenosis of a healthy organ is called 'eubiosis'. Different 'micro-biotopes', or localities, determine their individual types of microbiocenosis. They have consequently characteristic critera of eubiosis. It is only if these criteria of eubiosis are sufficiently definable that the criteria of an abnormal, disturbed status can be accurately expressed. A condition of disturbed microbiocenosis is termed dysbiosis, as proposed by Scheunert (1920).

It was not the aim of this work to distinguish, or even to look for, pathogenic microbes. Interest was focussed on ecological, not hygienic or epidemiological, problems. This can be illustrated with the example of typhoid-paratyphoid carriers. These carriers usually have a typical eubiotic faecal flora in which the salmonellae have become established as ecological partners. The pathogens play a normal part in the microbiocenosis along with other enterobacteriaceae and do not disturb the ecological balance.

3. METHODS

A weighed amount of the material under examination (e.g. stool, saliva, vaginal secretion, milk, cerumen, tears, semen, sweat, etc.) is decimally diluted in the range of 10^{-2} to 10^{-10}. Suitable dilutions are then cultivated on different media (nutrient-yeast agar with reducing agents; media without selective properties; tomato-sorbic acid agar at pH 5 for lactobacilli; SF-agar for enterococci; Endo-agar for Enterobacteriaceae; potato-glucose agar at pH 3·5 for fungi; mannitol-salt agar for staphylococci; urea-ricinoleate broth for *Proteus* spp.; egg yolk-neomycin agar for lecithinase positive clostridia; and egg white-liver nutrient broth for total putrefactive organisms). In addition to these media, others have been used more or less successfully.

Aerobic cultures on agar are prepared following the droplet method described by Miles & Misra (1938), anaerobic agar cultures are prepared either in deep layers in flat tubes (8×16×160 mm) or on agar plates by the droplet method. In the latter method the drops are covered with watch glasses of 40 mm diameter (Krüger, 1954), the bottoms of which contain 1 ml of nutrient agar inoculated with *Serratia marcescens* and preincubated at 37° for 6 hr before being applied over the drops. Fluid cultures (for *Proteus* spp. and putrefactive bacteria) are prepared in replicate and evaluated by statistical tables to give the most probable numbers. The genera *Fusobacterium* and *Veillonella* could only be cultured selectively from saliva with the media described by Omata & Disraely (1956) and Rogosa (1956). Detailed descriptions of the techniques used have been given elsewhere (Haenel, 1960*a*,*b*).

4. RESULTS

(a) *Eubiosis*

Life starts in a practically germ free state, since before birth the defence mechanisms of the mother usually prevent any contact of the unborn with micro-organisms. But during birth the newborn is exposed to an abundance of bacteria from the environment, mainly from the mother's vaginal and faecal microflora. Thus infection takes place very rapidly, and generally the first stool, the meconium, is no longer sterile

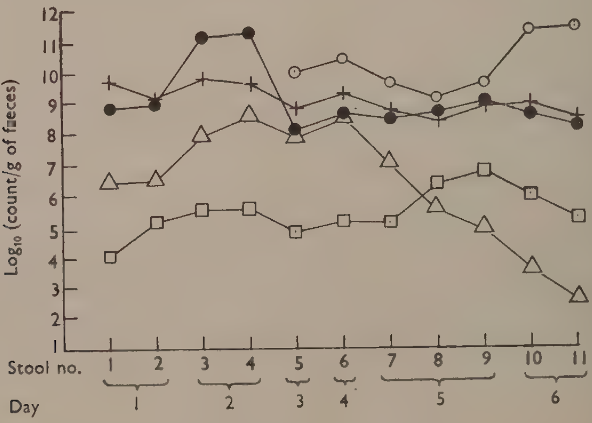

Fig. 1. The appearance of the different groups of bacteria in the meconium and stool of a breast fed newborn baby.
Open circles, anaerobic lactobacilli; full circles, aerobic lactobacilli; crosses, coli-aerogenes bacteria; triangles, putrefactive bacteria; squares, staphylococci.

(Schubert, 1958). The quality and quantity of the cultivable organisms are very diverse, and may change in the same infant from stool to stool. But under the influence of exclusive breast feeding the typical microbiocenosis develops within 3 to 6 days. The indications of this are high counts of anaerobic lactobacilli (for simplification called the 'bifidus' group), medium counts of coli-aerogenes bacteria, aerobic lactobacilli (called the 'acidophilus' group), enterococci and staphylococci, and—in our opinion the most important effect—a strong reduction of putrefactive bacteria (Fig. 1). Mother's milk is the only known food which suppresses putrefaction as well as putrefactive organisms in the colon (Feldheim, Schmidt & Haenel, 1960). It is supposed that the high bifidus counts are merely a result of this fact. However, this only applies to humans, for animal sucklings fed with their mother's milk do not show this type of microbiocenosis; putrefactive bacteria are always demonstrable, though putrefactive processes may be weak. The dominating lactobacilli are not obligate anaerobes (Haenel, 1960*a*).

Table 1. *A comparison of the counts of faecal organisms in an infant before and after weaning*

Group of micro-organisms	Log_{10} count/g of faeces		Mean count of population (10^6/g)	
	Before weaning (10 analyses)	After weaning (4 analyses)	Before weaning	After weaning
Anaerobic lactobacilli	9·9, 10·0, 10·0, 10·0, 10·1 10·1, 10·2, 10·3, 10·3, 10·5	9·5, 9·7, 9·7, 9·9	14,800	5,300
Aerobic lactobacilli	8·5, 8·6, 8·7, 8·8, 8·8 8·8, 8·9, 9·2, 9·3, 9·3	6·0, 6·8, 6·8, 7·1	980	6
Coli-aerogenes bacteria	7·9, 7·9, 7·9, 8·0, 8·2 8·9, 9·0, 9·2, 9·7, 9·7	8·8, 8·9, 9·2, 9·6	1,390	1,750
Enterococci	7·1, 7·5, 7·6, 7·8, 7·9 7·9, 7·9, 8·0, 8·1, 8·3	5·9, 6·7, 6·8, 7·2	85	7
Staphylococci	5·0, 5·6, 5·8, 5·8, 6·0 6·2, 7·2, 7·4, 7·8, 7·9	4·9, 5·3, 5·4, 5·5	18·5	0·2
Fungi	0·0	0·0, 0·0, 3·2, 3·2	0·0	0·008
Putrefactive bacteria	1·8, 2·4, 2·4, 2·4, 2·8 2·8, 2·8, 3·4, 3·4, 3·4	9·4, 9·4, 9·4, 9·4	0·001	2,500

During weaning the counts of putrefactive bacteria increase; bottle fed babies harbour numerous putrefactive organisms in their colons (Table 1). The anaerobic lactobacilli decrease only slightly. The counts of aerobic lactobacilli, coli-aerogenes bacteria, enterococci and staphylococci are either unchanged or changes are not reproducible (Feldheim, 1958; Haenel & Feldheim, 1958). It seems likely that the anaerobic lactobacilli which are dominant before weaning belong to a different group from those demonstrable after weaning (Dehnert, 1959). But the possibility cannot be excluded that with weaning only one group is suppressed, while others already present remain unchanged or increase in numbers. The relationships between the different groups of cultivable bacteria are similar in infants as in adults (Haenel, Schmidt & Feldheim, 1959; Haenel, 1960*a*). Anaerobic lactobacilli constitute more than 50% of the cultivable flora, and putrefactive bacteria about 20%. Other organisms generally occur in a minority of less than 1% and only rarely between 5% and 10%. Staphylococci and fungi are only intermittently demonstrable and then their counts are low. *Proteus* spp. are usually present, but in very low numbers of a few hundred or thousand/g of faeces (Haenel & Dawidowski, 1961).

In spite of the considerable variations in the bacterial counts, our results have demonstrated that the microbiocenosis of the colon in healthy persons is very constant. Experiments with normal and extreme diets (e.g. diets of meat and eggs, raw vegetables, vegetables, milk and vegetables), given for two weeks, showed only minor fluctuations which remained within the limits of the normal distribution. For example, an egg and meat diet or a milk diet sometimes increased the coli-aerogenes counts whereas hard cheese increased the counts of enterococci, but nevertheless the usual microecological relations were not changed (Haenel, Müller-Beuthow & Scheunert, 1957).

We were not able to confirm the assertion of Rettger & Cheplin (1921), that after lactose ingestion "*L. acidophilus* largely or entirely dominated the flora, effecting an almost complete suppression of all other bacterial types, commonly found in the enteric tract". In our own experiments Gram-stained smears of faeces showed a larger proportion of Gram-positive rods after the daily administration of 300 g of lactose, but the cultivable flora was hardly altered (Haenel, Feldheim, Müller-Beuthow & Ruttloff, 1958). Only lactulose—the 'bifidus factor' of Petuely (1957)—caused a change in the flora of the colon, mainly by reducing the putrefactive bacteria without affecting the bifidus group: but these changes varied directly with the extent of diarrhoea produced. These observations that extreme diets are ineffective in changing the faecal flora agree with those of Torrey & Montu (1931).

Table 2. *The incidence of different groups of micro-organisms in the saliva of adults**

Group	No of analyses* negative	Frequency of occurrence of counts (ml of saliva) in the ranges				
		$<10^3$	10^3–10^5	10^5–10^7	10^7–10^8	10^8–10^9
Total organisms	0	0	0	0	10	10
Lactobacilli	0	0	0	9	8	3
Putrefactive bacteria	0	0	0	13	7	0
Staphylococci	2	9	9	0	0	0
Fungi	13	2	5	0	0	0
Proteus spp.	11	9	0	0	0	0
Veillonella spp.	0	0	0	15	5	0
Fusobacterium spp.	0	0	1	18	1	0

* Twenty analyses were done on 10 adults.

Investigations have been carried out to discover whether the different parts of the intestinal canal give rise to different microbiocenoses. As expected, the flora of the mouth shows a distinct pattern (Table 2), which is in agreement with the thesis that an organ with special anatomical or physiological properties would form a typical and distinct flora (Haenel, Feldheim & Müller-Beuthow, 1958). Post mortem investigations confirmed the findings of Cregan & Hayward (1953) and others that the unaffected small intestine is almost sterile. Only the lower ileum may contain a microbiocenosis, similar to that in the colon but with fewer organisms. Within the colon the microbiocenosis remains constant, and has the same composition as the faecal flora (Haenel & Müller-Beuthow, 1958).

(b) *Dysbiosis*

It follows from the foregoing that the criteria of an eubiotic intestine are the lack of micro-organisms in the small intestine, and a cultivable flora in the colon consisting predominantly of anaerobic lactobacilli, accompanied by a certain proportion of putrefactive bacteria and a minority of other organisms, mostly coli-aerogenes bacteria and enterococci. In the following investigations the criteria, occurrence and frequency of an abnormal, dysbiotic flora were investigated.

When a eubiotic stool was preserved at 4° its bacterial composition remained unchanged for 3 to 6 days. Thereafter the anaerobic lactobacilli disappeared whereas enterococci, and to a lesser, variable, extent coli-aerogenes bacteria and *Proteus* spp. increased (Haenel & Müller-Beuthow, 1959).

Dysbiotic findings were very frequent in suckling infants convalescing in hospital. The same was found in infants thriving poorly, or in infants with slight infections (e.g. infections of the throat), but who were otherwise healthy. Healthy children (at home or in nurseries) always had eubiotic conditions in the intestine. The indications of dysbiosis were similar: absence, or reduction, of the anaerobic lactobacilli and increases in coli-aerogenes bacteria, enterococci, *Proteus* spp., and sometimes other micro-organisms. The results gave the impression that the factors ruling the composition of intestinal microbiocenoses in young children are easily influenced or disturbed (Haenel, Schmidt & Feldheim, 1959).

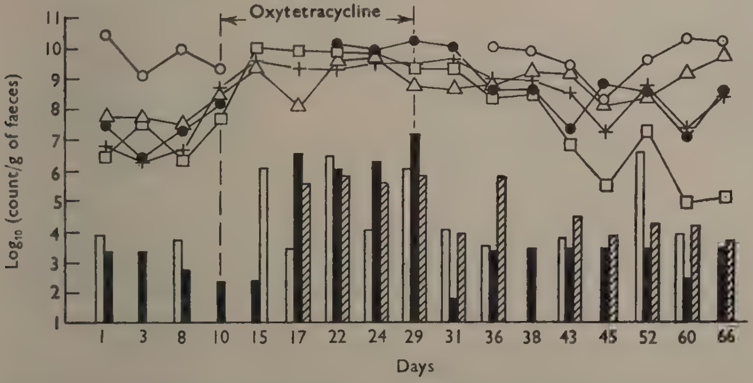

Fig. 2. The influence of oxytetracycline, administered for 19 days at a dose of 250 mg/4 hr, on the faecal microbiocenosis of a paratyphoid carrier. Open circles, anaerobic lactobacilli; full circles, aerobic lactobacilli; triangles, putrefactive bacteria; crosses, coli-aerogenes bacteria; squares, streptococci; plain areas, fungi; solid areas, *Proteus* spp.; hatched areas, staphylococci.

Experiments on typhoid-paratyphoid carriers with tetracycline therapy resulted in a prompt change from eubiotic to dysbiotic relations within the faecal flora, although without any clinical disturbances. Fig. 2 shows the disappearance of anaerobic lactobacilli from the stool during therapy with oxytetracycline, with a corresponding increase in other groups of bacteria. A few days after the antibiotic was withdrawn, the eubiotic flora re-established itself (Haenel, Köhler, Mertsch & Pardemann, 1960).

Table 3. *The incidence of different groups of bacteria in the faecal flora of healthy adults**

Group	No of analyses* negative	Frequency of occurrence of counts (/g of faeces) in the ranges						
		$<10^3$	10^3–10^5	10^5–10^7	10^7–10^8	10^8–10^9	10^9–10^{10}	$>10^{10}$
Anaerobic lactobacilli	0	0	0	0	0	5	39	6
Aerobic lactobacilli	8	0	3	15	18	6	0	0
Coli-aerogenes bacteria	0	0	0	4	19	22	5	0
Enterococci	0	0	5	24	21	0	0	0
Proteus spp.	0	7	41	2	0	0	0	0
Clostridia	6	8	28	8	0	0	0	0

* Fifty analyses were done on 21 adults.

Table 4. *The incidence of different groups of bacteria in the faecal flora of gastrectomized patients**

Group	No of analyses*		Frequency of occurrence of counts (/g of faeces) in the ranges						
	Total	Negative	$<10^3$	10^3–10^5	10^5–10^7	10^7–10^8	10^8–10^9	10^9–10^{10}	$>10^{10}$
Anaerobic lactobacilli	62	48	0	0	0	0	3	10	1
Aerobic lactobacilli	62	29	0	0	6	6	11	10	0
Coli-aerogenes organisms	62	0	0	0	0	4	7	45	6
Enterococci	52	0	0	1	6	13	19	13	0
Proteus spp.	62	2	5	25	29	1	0	0	0
Clostridia	41	3	1	4	22	10	1	0	0
Atypical germs	62	42	0	0	0	2	3	13	2

* Analyses done on 21 patients.

In Tables 3 and 4 the analyses of stools of normal and gastrectomized persons are compared. The criteria of dysbiosis appearing after gastrectomy were similar to those mentioned above: a loss of anaerobic lactobacilli combined with an increase of coli-aerogenes bacteria, enterococci, *Proteus* spp., clostridia and atypical bacteria (aerobic sporeformers, etc.). Only occasionally was dysbiosis observed in other types of disease, possibly connected with a bad condition of the patient rather than directly with the disease (Haenel & Feldheim, 1961). But further investigations are needed to clarify this question. According to these results the microecological relations in the adult are more stable and resistant than those in young infants: they do not seem to be easily disturbed.

The results of investigations conducted on material from the jejunum (taken 1 m above the ileocaecal valve) and colon (taken 70 cm below the ileocaecal valve) are summarized in Table 5. Contents of these intestinal parts were obtained from patients during appendectomy by inserting a syringe needle. The total contents of the syringe were examined: hence the range of volume shown in Table 5. These results prove the connection between a eubiotic flora of the colon and a sterile jejunum, and between a dysbiotic flora of the colon and high bacterial counts in the jejunum. These conditions of a dysbiotic colon again agree with the results mentioned above (Haenel, von Lutzki & Feldheim, 1961).

Table 5. *The eubiotic and dysbiotic microecology of the intestinal canal of adults determined by examining samples obtained by puncture during appendectomy*

Condition and no. of analyses	Situation*	Presence†, or no.‡ of organisms, of groups							
		Anaerobic lacto-bacilli	Aerobic lacto-bacilli	Gram −ve anaerobes	Putre-factive bacteria	Coli-aerogenes bacteria	Entero-cocci	Clostridia	Other organisms
Eubiotic 15	A	—	(+)	—	(+)	—	(+)	—	(+)
	B	1,600	2	70	10	19	1	(+)	—
Dysbiotic 5	A	—	4	(+)	20	280	6	(+)	440
	B	—	48	1,600	260	1,000	380	8	1,500

*. A, jejunum 1 m above ileocaecal valve; B, colon 70 cm below ileocaecal valve.

† —, absent; (+), occasionally present.

‡ Expressed as millions/0·5–0·05 ml of intestinal contents.

5. Conclusions

According to our results the microbiocenosis of an organ is a well defined, reproducibly composed, ecological system consisting of the macro-organism and certain distinct kinds of micro-organisms. Anatomy, physiology, and, to a lesser degree hygiene, form and influence the conditions in an organ and are responsible for the typical composition of the microbiocenosis. The techniques available do not permit the complete composition of the microbiocenosis to be determined, but only some cultivable parts of it. The results can therefore be considered only as indicators of the state of affairs existing. They point to the rules which may govern the micro-ecological aspects of man's existence. They give some hints about the consequences of these rules, but do not reveal their causes.

6. Summary

The macro-organism and its microflora form a balanced ecological system. According to their anatomical, physiological and hygienic characteristics the different organs constitute distinct microenvironments and are inhabited by typical microbial associations (microbiocenoses). The condition in a normal, naturally occurring, reproducibly composed, microbiocenosis of a healthy organ is called eubiosis, in contrast to dysbiosis, which term denotes a disturbed microflora. The eubiotic small intestine is almost sterile, the typical colon flora developing near the ileocaecal valve. The microfloras of different parts of the colon and of the faeces are similar and the cultivable parts of this flora consist mainly of anaerobic lactobacilli (more than 50%) the second highest group being putrefactive bacteria, mainly *Bacteroides* spp. Coli-aerogenes bacteria, enterococci and other organisms are in a minority (usually less than 1%).

The composition of this flora remains very stable, extreme diets producing only minor changes or none at all. In breast fed babies putrefactive bacteria are absent, otherwise the microecological relations are similar.

The criteria of dysbiosis are: a reduction or elimination of anaerobic lactobacilli and an increase of other organisms, mainly coli-aerogenes bacteria, enterococci, *Proteus* spp., clostridia and others. Simultaneously the small intestine appears to become inhabited by bacteria from the colon. Dysbiotic conditions were frequently found in sick, convalescent or poorly thriving infants, in adults during therapy with tetracyclines, and after gastrectomy; they were less frequent with the other diseases so far examined.

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STUDIES ON THE BACTERIAL FLORA OF THE ALIMENTARY TRACT OF PIGS. I. ENTEROBACTERIACEAE AND OTHER GRAM-NEGATIVE BACTERIA

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1. Introduction

Several workers have regularly obtained counts on media selective for members of the Enterobacteriaceae from samples of the faeces and intestinal contents of pigs, but there is little information on the identity of the component groups present. In no instance have members of other families of Gram-negative facultative anaerobes been isolated from apparently healthy pigs.

On the basis of dilution counts made in MacConkey's broth, Willingale & Briggs (1955) calculated that, for 22 pigs receiving a 'standard' ration for fattening, the average numbers of coli-aerogenes organisms present/g of wet material were $4{\cdot}2 \times 10^6$ in the caecal contents and $7{\cdot}31 \times 10^6$ in the faeces. In a subsequent publication on the effect of dietary supplements on the faecal flora of the pig, Fuller, Newland, Briggs, Braude & Mitchell (1960), using the same quantitative method, confirmed these figures for faeces of control animals. Isolates from the highest positive MacConkey's broth tubes were identified by IMViC and Eijkman tests as *Escherichia coli* faecal type I and those from tubes showing acid only as *E. coli* Irregular type I. Horvath, Seeley, Warner & Loosli (1958), using the same medium in studies on the influence of the diet on the intestinal microflora of pigs, reported average counts of 1×10^8/g in the stomach and 1×10^{10}/g in the small and large intestine and faeces of animals fed a 'basal' corn-soybean oil-meal diet for growing and fattening. *Proteus* spp. were estimated at approximately 1×10^4/g with the selective medium of Zarett & Doetsch (1949), but difficulties were encountered in the use of this medium and it was not regularly employed. During a study on the effect of dietary antibiotic supplements on the intestinal microflora of pigs, Bridges, Dyar & Powers (1953) obtained average counts from the faeces of control animals of 1×10^6/g in Bacto violet red-bile agar, $3{\cdot}7 \times 10^7$/g in Bacto urea agar and $3{\cdot}9 \times 10^7$ in Bacto brilliant green agar, which they considered represented coli-aerogenes organisms, *Proteus* spp. and *Shigella* strains, respectively. Quinn, Story, Catron, Jensen & Whalen (1953), using Bacto deoxycholate agar during their studies on the effect of antibiotics on the intestinal flora, obtained an average count in the faeces of $1{\cdot}15 \times 10^7$/g of wet material for pigs on a high protein diet and $3{\cdot}35 \times 10^7$/g for those on a low protein diet. In a subsequent publication (Quinn, Lane, Ashton, Maddock & Catron, 1953) they reported counts obtained at weekly intervals from control animals of 2×10^6 (3 pigs), 3×10^6 (2 pigs), 135×10^6 (2 pigs) and 31×10^6 (4 pigs)/g of wet faeces, with Bacto violet red-bile agar and eosin–methylene blue agar. The pigs examined in these experiments received a 'basal' corn-soybean ration. Recently Kjellander (1960) counted the "typically yellow colonies of *E. coli*" which developed after pads used for filtering the 10^{-3} to 10^{-6} dilutions of 12 samples of pigs' faeces had been incubated on a modification of Conradi-Drigalski agar. He obtained an average count of $3{\cdot}9 \times 10^6$, the limits being $1{\cdot}9 \times 10^5$ and 21×10^6/g of wet faeces.

The object of the present work was to identify the dominant component groups of Gram-negative facultative anaerobes enumerated on Bacto deoxycholate agar (Difco) from samples of the faeces of pigs which were receiving a standard diet for fattening, and from samples of their gastric, intestinal and caecal contents at slaughter. Animals receiving dietary antibiotic supplements were included in the experiments. As the work was carried out in conjunction with studies on the entire bacterial flora, isolations were sometimes made from other media for comparison. Also, in some cases, organisms in the subdominant flora were identified. Isolates of Enterobacteriaceae were differentiated and named in accordance with the descriptions given in Report (1958), with certain modifications in the bacteriological tests employed.

2. MATERIALS AND METHODS

All concentrations are given as % (w/v) unless stated otherwise.

(a) *General*

Pigs. The animals studied were of the Large White and Piétrain breeds and weighed between 20 and 100 kg. They were housed in individual pens and reared under commercial conditions in the piggeries of the C.N.R.Z. at Jouy-en-Josas.

Rations. The rations received by the animals are given in Table 1. Dietary supplements of spiramycin, aureomycin, erythromycin, neomycin and bacitracin (20 mg/kg of ration A) were received by some of the animals.

Table 1. *The composition of rations received by pigs during the experiments*

Ration	Composition (% w/w) of ration								
	Barley	Wheat	Groundnut cake	Soyabean cake	Fish powder	Wheat chaff	Meat powder	Yeast	Mineral mixture
A	38	38	4	4	2	5	2	4	3
	Barley	Maize	Crude soya flour	Fish powder	Meat powder	Milk powder	Sorghum	Millet	Mineral mixture*
B	38·5	15	6	3	4	3	15	12·5	3
C	Ration A plus kitchen waste								

* This mineral mixture also included vitamins A, B_1, B_{12}, D_3, nicotinic acid and pantothenic acid.

Samples. Gastric, intestinal and caecal contents collected directly after the carcases had been plunged into hot water after slaughter by electroshock, and fresh faeces, were taken to the laboratory and examined immediately. The animals were slaughtered in the abattoir at the C.N.R.Z. approximately 16 hr after they had received the last feed. Manipulation of the samples in the laboratory was completed within the 2 hr following death.

Preparation for bacteriological examination, and isolation. Decimal dilutions in 9 ml quantities of a meat extract broth (Lab-Lemco, 0·5%; peptone (Evans), 0·5%; Bacto tryptone (Difco), 1·0%; in deionized water; pH 7·4–7·5) were made from a 10^{-1} dilution prepared by mixing 50 g of the sample in 450 ml of distilled water in a 'Virtis' macerator at 14,000 rev/min. Quantities of 1 ml of the dilutions were poured with Bacto deoxycholate agar (DCA) in Petri dishes and incubated aerobically for 24–48 hr at 37°. Isolates from representative colonies developing on the highest dilution plates were generally selected for the bacteriological tests to be described. Incubation was at 30° except where mention is made to the contrary.

(b) *Methods for a primary classification of strains*

Determination of oxygen requirements and glucose fermentation. Stab inoculations were made into freshly boiled and cooled tubes containing 6–8 ml of a glucose agar (GA) medium. This contained: Bacto peptone (Difco), 0·5%; Lab-Lemco (Oxoid), 0·5%; glucose, 0·5%; agar, 1·5%; 0·6% alcoholic bromocresol purple, 0·1–0·15% (v/v); in deionized water. The pH was adjusted to 6·8–7·0. Acid and gas production and subsequent 'reversal' of the indicator colour from yellow to purple at the surface were noted over a period of 3–4 days.

Inoculations for the following tests were made from 24 hr cultures in the GA medium.

Lactose fermentation. This was determined over a period of 14 days in 'ordinary' peptone water (PW) (Cunningham, 1947, p. 39) containing 0·5% of lactose and bromocresol purple as indicator. Fermentation occurring within 24 hr was considered to be prompt, whereas if it occurred after 24 hr it was classed as delayed.

Indole production. This was determined on cultures in a medium containing 1% of tryptone (Difco) and 0·5% of K_2HPO_4 after incubation for 5 days, either by the Erhlich-Böhme test (Wilson & Miles, 1948) or a modification of the xanthydrol test (Fearon, 1944). In the latter case, tubes containing 0·5 ml of the culture and 1 ml of 1% xanthydrol in acetic acid were examined after 2–10 min in boiling water. A deep or pale purple colour indicated the presence of indole.

Lysine decarboxylation. The mineral salts medium containing 0·2% of L-lysine monohydrochloride described by Carlquist (1956) with 0·1% of yeast extract (Difco) was sterilized in 5 ml quantities at 115° for 20 min. The ninhydrin test was carried out as described by Carlquist after incubation at 37° for 18–24 hr.

Growth and lactose fermentation at 45° in MacConkey's bile salt broth. These were determined over a period of 14 days in the medium described in Report (1949).

(c) *Methods for further differentiation*

Inoculations were made from 24 hr cultures on nutrient agar slopes containing: Lab-Lemco, 0·5%; peptone (Evans), 1·5%; Bacto tryptone (Difco), 1·0%; agar, 2·0%; in deonized water; pH 7·4–7·5.

Gelatin liquefaction, choline and urea destruction and indole production. The following 'combined' medium was used. It contained: peptone (Evans), 0·5%; Bacto tryptone (Difco), 1·0%; Lab-Lemco (Oxoid), 0·5%; choline chloride, 0·5%; urea, 0·2%; gelatin, 1·2%; in deionized water. The first three ingredients were dissolved in the water and the pH adjusted to 7·7. After heating at 120° for 20 min and filtration the other ingredients were added and the medium tubed in 4–5 ml quantities and sterilized at 115° for 20 min. Incubation was at 37° for one week. Gelatin liquefaction was noted after holding at 4° for 10–15 min. The presence of choline was detected with a modified Nessler's reagent composed of 27·1 g of finely powdered $HgCl_2$ (analytically pure) and 36·0 g of KI (analytically pure) in 1,000 ml of distilled water. A heavy yellow precipitate occurs in the presence of choline, whereas there is no reaction when the choline has been destroyed. A volume of 1 ml of the xanthydrol reagent described above was added to 0·5 ml of the culture to test for the presence of urea and indole. The formation of a heavy crystalline precipitate after 1 min in boiling water is indicative of urea, but when this has been hydrolyzed the contents of the tube are clear or show a less dense precipitate of a more granular nature. The colour reaction for indole was noted after a further 2–10 min in boiling water.

Gelatin liquefaction and H_2S production at 22°. These characters were determined according to the methods described in Report (1958).

Urease production. This was also determined with both Christensen's urea agar medium (Report, 1958) and the urea medium of Hormaeche & Munilla (1957) incubated at 37° for 48 hr.

Detection of acetoin and reaction to methyl red. A semisynthetic medium based on that of Billen & Lichstein (1950) and containing: KH_2PO_4, 0·1%; $MgSO_4.7H_2O$, 0·07%; NaCl, 0·1%; $(NH_4)_2HPO_4$, 0·4%; $FeCl_3$, 0·00005%; glutamic acid, 0·5%; yeast extract (Difco), 0·1%; in distilled water; pH 6·8, was tubed in 4 ml quantities and autoclaved at 115° for 20 min. To each tube was added 1 ml of a 2·5% glucose solution sterilized separately. Tests were carried out after incubation for 3 and 4 days. For acetoin production (V-P reaction), 1 ml of 0·6% α-naphthol in 95% alcohol and 1 ml of 40% KOH solution containing 2·5% of creatine were added to 1 ml of culture. The colour reaction was noted 10–20 min after vigorous shaking. To a further 1 ml of culture 0·5 ml of a methyl red solution (Report, 1949) was added (M.R. test).

Oxidative deamination of phenylalanine. A basal mineral salts medium (Shaw & Clarke, 1955) containing 0·1% of yeast extract (Difco) with 0·2% of DL-phenylalanine was tubed in 5 ml quantities and sterilized at 115° for 20 min. Cultures in this medium were incubated for 24 hr at 37° and the presence of phenylpyruvic acid detected by the ferric chloride test (Shaw & Clarke, 1955) and the '2:4-D' test, a modification of the technique described by Friedemann (1957). In the latter test 1 ml of 0·1% 2:4-dinitrophenylhydrazine in 2 N HCl and 2 ml of 1·5 N NaOH were added to 1 ml of the culture. The development of a red colour indicates a positive reaction.

Breakdown of arginine. This was determined in an arginine medium (AM) composed of the basal mineral salts-yeast extract medium used for the decarboxylation of lysine (see above), without $(NH_4)_2SO_4$, and containing 0·2% of L-arginine monohydrochloride. Duplicate tubes were inoculated, and paraffin oil was layered on one of them to give anaerobic conditions. After incubation for 4 days, NH_3 was detected with Nessler's reagent on a 'spot-plate'. Ornithine was tested for with the ninhydrin reagent described by Chinard (1952) by the following method. A tube containing 0·1 ml of the culture, 0·4 ml of distilled water, 1·5 ml of acetic acid and 0·5 ml of the ninhydrin reagent was heated in boiling water for 20 min. A brownish red colour indicates the presence of ornithine. Keto compounds were detected by the '2:4-D' test described above.

Ammonia with ornithine indicates breakdown by the arginine dihydrolase system (Oginsky, 1955), and NH_3 with keto compounds indicates oxidative deamination.

Arginine and ornithine decarboxylation, and production of arginine dihydrolase. The first two of these tests were done according to the methods of Møller (1955) and the last by the method of Hormaeche & Munilla (1957).

Malonate utilization. This was determined over a period of 5 days in the basal mineral salts medium described by Clapper & Poe (1947), to which were added 0·1% of malonic acid and bromothymol blue as indicator.

Utilization of ammonium citrate. This was determined over a period of 5 days in a medium similar to Simmons' citrate medium but containing no agar (Cunningham, 1947, p. 34).

Citrate utilization on modified Christensen's agar. The medium modified by Edwards & Ewing (1955) was used for this test.

Fermentation of carbohydrates other than starch. This was determined either in peptone water or in a peptone meat extract medium (PM) containing: Bacto peptone (Difco), 1·0 g; Lab-Lemco, 0·5 g; NaCl, 0·3 g; Na_2HPO_4, 0·2 g; in 100 ml of deionized water; pH 7·0; with, in each case, bromocresol purple as indicator. Seitz-filtered solutions of suitable carbohydrates were added to the sterile medium to give a concentration of 0·5%.

Fermentation of insoluble starch. This was determined according to the method given in Report (1958).

Nitrate reduction. Tests for nitrate reduction were done according to Report (1958).

Cytochrome oxidase production. This was determined by flooding 24 hr nutrient agar slope cultures with 1% aqueous *p*-aminodimethylaniline oxalate (Difco) (Manual, 1953).

Motility and sucrose fermentation. These were determined in the medium described in Report (1958), to which were added 0·5% of sucrose and bromocresol purple as indicator.

Catalase production. This was determined by adding a loopful of a nutrient agar slope culture to a drop of H_2O_2 (10 vol) on a slide. If the result was negative a tube test was carried out with 1 ml of a culture in tryptone medium and 0·5 ml of 10 vol H_2O_2.

Serological methods for Enterobacteriaceae. Agglutination tests were carried out on slides by the method described by Kauffmann (1951) and in tubes by the centrifugation method (Le Minor, pers. comm.). Sera for *Escherichia* strains were kindly sent by Dr. Le Minor and Dr. Ørskov.

Additional media for groups other than Enterobacteriaceae. Brucella agar (Albimi Laboratories Inc.), sheep's blood agar—prepared by adding 5% of citrated sheep's blood to Bacto blood agar base (Difco)—potato slopes, coagulated serum and Löffler's coagulated serum (obtained, ready prepared, from the Institut Pasteur, Paris) were used. In some cases the medium (LAPT) described by Raibaud, Caulet, Galpin & Mocquot (1961) was used as the basal medium for carbohydrate fermentation (with bromocresol purple as the indicator) and for aesculin hydrolysis (Cunningham, 1947, p. 36).

3. RESULTS

(a) *Preliminary observations*

Only those strains showing a facultative oxygen requirement in the GA medium were selected for the bacteriological tests outlined. A provisional grouping of these strains could be made on the results of the tests for the primary classification (Table 2).

Gram-negative strict aerobes were isolated from time to time, particularly from gastric contents. These formed easily recognizable small, lenticular, rose coloured deep colonies and small, opaque, mucoid, rose or transparent yellow surface colonies on DCA plates.

Most of the lactose positive, indole positive group I strains were subjected only to the tests outlined for the primary classification. Strains of all groups selected for further study by the methods outlined for further differentiation were checked for purity by streak inoculation on DCA plates.

Group I strains were always present and invariably formed the dominant flora on DCA plates, whereas other groups of Enterobacteriaceae appeared only from time to time on high dilution plates. These observations have been reported in a previous publication (Dickinson, 1961) in which it was also shown that there was no difference in the distribution of Enterobacteriaceae groups in the floras of the alimentary tracts and faeces of control animals and animals receiving dietary antibiotic supplements. For this reason it was decided to include in this report isolates originating from animals fed with antibiotics in addition to those from control animals.

Table 2. *The primary differentiation of Gram-negative facultative anaerobes isolated from the alimentary tract of pigs, into groups*

Reaction* in GA medium	Reactions* in tests for					Group
	Lactose fermentation		Indole formation	Lysine decarboxylation	Growth at 45° §	
	30°	45° §				
AG or A	AG, A, x or —	AG, A	d	d	+**	I, II, III
AGr	AG, x, or —	—	—†	d	—	IV, V, VI
ag or a	—	—	d	—	—†	VII, VIII
(a)‡ or (ag)‡	— or a‡	—	—	—	—	IX, X

* Reactions: +, positive; —, negative; A, acid; G, gas; a, slight acid; (a), very slight acid; g, slight gas; (g), small bubble of gas at surface of GA medium; r, 'reversal' of indicator at surface of GA medium; x, slow fermentation with acid and gas production; d, different biochemical types. † Rarely positive. ‡ Examined after 1–2 days. § Tested in MacConkey's broth. ** Rarely negative.

(b) *Description of the strains isolated and their differentiation into groups*

(i) *Enterobacteriaceae*

The reactions of all the groups differentiated are given in Tables 3 and 4. Differing reactions observed in certain of the bacteriological tests will be given in more detail in the text.

Group I. Type a strains (1,115 isolates) showed prompt lactose fermentation at 30°. The reactions were typical for *Escherichia* strains (Report, 1958). Variations were observed for indole production and lysine decarboxylation (Table 5) and production of acid and gas from lactose in MacConkey's broth at 45°, although the majority of strains gave positive reactions in these tests. Choline was not destroyed in the 'combined' medium. Of 130 strains tested for cellobiose fermentation, 128 were negative and 2 fermented the sugar after 3 days.

Group I, type b strains (184 isolates) showed delayed lactose fermentation at 30°. Reinoculation of these strains on to DCA plates resulted in the production of 'red' lactose fermenting colonies and 'yellow' lactose non-fermenting colonies in 24 hr, or of red papillae after 2–3 days. Further subculture of these papillae resulted in the production of 'red' and 'yellow' colonies. Substrains from 'red' and 'yellow' colonies gave identical reactions in the other differential tests even though the 'red' substrains fermented lactose promptly and the 'yellow' substrains showed a delayed fermentation of the sugar. This behaviour is typical of '*Bacillus coli mutabilis*' (Neisser, 1906). A total of 79 of the type b strains destroyed choline. All the choline positive strains tested (43) fermented cellobiose, whereas the choline negative strains tested (70) did not do so. None of the choline positive strains fermented lactose at 45° in bile salt broth; but 66 of the choline negative strains fermented it slowly at 45°. Strains of type b may be designated as slow lactose fermenting strains of the *Escherichia* group (Report, 1958).

Group I, type c strains (195 isolates) did not ferment lactose at 30° (Table 3) and only 48 of them destroyed choline (Table 5). The 36 choline positive strains tested for cellobiose fermentation were positive, whereas all choline negative strains tested (41) were negative. All the choline positive strains tested were unable to ferment lactose at 45° in bile salt broth, although 14 choline negative strains out of the 41 tested fermented lactose under these conditions. Since type c strains did not ferment lactose at 30° they may not be classified in the *Escherichia* group (Report, 1958);

Table 3. *The biochemical and cultural characters of Enterobacteriaceae of ‘groups’ I, II and III from the alimentary tract of pigs*

Test		Biochemical and cultural characters* and number of isolates of ‘group’						
		I					II	III
		Type a (1,115)	Type b (184)	Type c (195)	Type d (91)	Type e (3)	(3)	(2)
Colonial appearance‡ on DCA plates	Surface	L, R	L, R or Y P	L, Y	as Ia	as Ic	as Ib or c	as Ib or c
	Deep	L, R, R-Z	R, M or S	R, M or S, Y-H	as Ia	as Ic	as Ib or c	as Ib or c
Reaction in GA medium		AG	AG	AG	A	A or AG	AG	AG
Lactose fermentation (30°)		AG	▲	–	A	–	▲	▲
Indole production		d	d	d	+	–	–	–
Lysine decarboxylase		d	d	d	+	–	–	–
MacConkey’s broth (45°)	Growth	+	+	+	+	–	–	–
	Lactose fermentation	AG	d	d	A	–	–	–
‘Combined’ medium (37°)	Gelatin liquefaction	–	–	–	–	–	–	+
	Choline destruction	–	d	d	–	–	–	–
	Urea destruction	–	–	–	–	–	d	–
	Indole production	d	d	d	+	–	–	–
$FeCl_3$–gelatin medium (22°)	Gelatin liquefaction	–	–	–	–	–	–	+
	H_2S production	–	–	–	–	–	–	–
Urease	(Christensen)	–	–	–	–	–	d	(×)
	(Hormaeche & Munilla) (37°)	–	–	–	–	–	–	–
V-P reaction		–	–	–	–	–	–	–
M.R. reaction		+	+	+	+	+	+	+
Phenylalanine deaminase		–	–	–	–	–	–	–
NH_3 from arginine§	aerobically	–	–	–	–	–	–	+
	anaerobically	–	–	–	–	–	–	–
‘2:4-D’ reaction§		–	–	–	–	–	–	–
Ornithine formation§		–	–	–	–	–	–	–
Arginine dihydrolase		–	–	–	–	–	–	–
Arginine decarboxylase		d	d	d	d	+	–	–
Ornithine decarboxylase		d	d	d	d	+	–	+
Malonate utilization		–	–	–	–	–	d	–
Ammonium citrate utilization		–	–	–	–	–	+	–
Citrate utilization (Christensen)		d	d	d	+	–	+	+
Fermentation of	Cellobiose	– or ▲†	AG or –	AG or –	–	–	AG or –	AG
	Mannitol	AG	AG	AG	A	AG or ▲	AG	AG
	Maltose	AG or –†	AG or –†	AG or –†	A or –†	AG or ▲	AG	AG
	Sucrose	AG or –†	AG or –†	AG or –†	A or –†	–	AG or –	AG
Gas from insoluble starch		–	–	–	–	–	–	–
Nitrate reduction		+	+	+	+	+	+	+
Cytochrome oxidase		–	–	–	–	–	–	–
Motility		d	d	d	d	–	+	+
Name given to group		*Escherichia*					*Citrobacter*-like	‘Unclassified type a’

* Characters: +, positive reaction; ×, slow reaction; ▲, slow fermentation with acid and gas production; ( ), weak reaction; –, negative reaction; d, different biochemical types; A, acid; G, gas; a, slight acid; g, slight gas; †, rare.

‡ Colonial appearance: L, large; M, medium; P, papillae; R, red; R-Z, surrounding red zone; S, small; Y, yellow; Y-H, yellow halo. All the deep colonies were lenticular; § Arginine medium AM.

Table 4. *The biochemical and cultural characters of Enterobacteriaceae of 'groups' IV, V, VI, VII and VIII from the alimentary tract of pigs*

Test		Biochemical and cultural characters* and number of isolates of 'group'					
		IV		V	VI	VII	VIII
		Type a (6)	Type b (3)	(29)	(6)	(23)	(10)
Colonial appearance‡ on DCA plates	Surface	L, m, R-Y	as IVa	L, R-Y	as Ic or V	L, F, T	as VII
	Deep	M, R, Y-H	as IVa	as IVa	as IVa	S, Y-H	as VII
Reaction in GA medium		AGr	AGr	AGr	AGr	ag or a	ag or a
Lactose fermentation (30°)		AG	AG or ▲	AG or ▲	AG or −	−	−
Indole production		−	d	−	−	d	△
Lysine decarboxylase		+	+	−	−	−	−
MacConkey's broth (45°)	Growth	−	d	−	−	d	−
	Lactose fermentation	−	A or −	−	−	−	−
'Combined' medium (37°)	Gelatin liquefaction	d	d	d	−	d	−
	Choline destruction	−	+	−	−	+	+
	Urea destruction	+	+	d	−	+	−
	Indole production	−	−	−	−	d	−
$FeCl_2$–gelatin medium (22°)	Gelatin liquefaction	−	d	−	−	d	(×) or −
	H_2S production	−	(+) or −	−	−	+ or (+)	(×) or +
Urease	(Christensen)	+	+	+	−	+	−
	(Hormaeche & Munilla) (37°)	+	+	−	−	+	−
V-P reaction		+	+	+	+	d	−
M.R. reaction		−	−	−	−	+ or (+)	(+)
Phenylalanine deaminase		−	−	−	−	+	+
NH_3 from arginine§	aerobically	+	+	+	−	+	+
	anaerobically	−	−	+	−	−	−
'2:4-D' reaction§		−	−	−	−	+	+
Ornithine formation§		−	−	+	−	−	−
Arginine dihydrolase		−	−	+	−	−	−
Arginine decarboxylase		−	−	+	d	−	−
Ornithine decarboxylase		−	−	+	+	d	−
Malonate utilization		+	+	+	d	−	−
Ammonium citrate utilization		+	+	+	+	d	+
Citrate utilization (Christensen)		+	+	+	+	d	+
Fermentation of	Cellobiose	AG	AG	AG	AG	−	−
	Mannitol	AG	AG	AG	AG	d	−
	Maltose	AG	AG	AG	AG	d	−
	Sucrose	AG	AG	AG	AG	d	×
Gas from insoluble starch		+	+	−	−	−	−
Nitrate reduction		+	+	+	+	+	+
Cytochrome oxidase		−	−	−	−	−	−
Motility		−	−	+	+	+	+
Name given to group		*Klebsiella*		*Cloaca* (*Aerobacter*)	'Unclassified type b'	*Proteus*	Providence

* Characters: +, positive reaction; △, positive, becoming negative after 24 hr; ×, slow reaction or slow fermentation with acid production; ▲, slow fermentation with acid and gas production; ( ), weak reaction; −, negative reaction d, different biochemical types; A, acid; G, gas; r, 'reversal' of indicator; a, slight acid; g, slight gas.

‡ Colonial appearance: F, flat; L, large; M, medium; m, mucoid; R, red; R-Y, red becoming yellow; S, small; T, transparent; Y-H, yellow halo. All the deep colonies were lenticular. § Argenine medium AM.

Table 5. *The behaviour of group I* (Escherichia) *isolates from the alimentary tract of pigs in certain tests*

Test	The ratio of positive to negative reactions in the indicated tests for isolates of				
	Type a	Type b	Type c	Type d	Type e
Indole production	1,038 : 77*	181 : 3	189 : 6	88 : 3	0 : 3
Lysine decarboxylase	1,108 : 7	182 : 2	69 : 26§	91 : 0	0 : 3
Choline destruction	0 : 197	79 : 105	48 : 147	0 : 11	0 : 3
Cellobiose fermentation	2† : 128	43‡ : 70§	36‡ : 41§	0 : 11	0 : 3

* Of these isolates, all produced lysine decarboxylase; of the proportion (35) of these included in the remaining tests all failed to destroy choline or ferment cellobiose.

† Fermentation at 3 days. ‡ Choline positive isolates. § Choline negative isolates.

but neither do they comply with the descriptions for lactose non-fermenting groups. The authors are of the opinion that these strains may eventually be shown to be members of the *Escherichia* group and so they have been provisionally included here, although for the present they should be considered as members of the genus *Paracolobactrum* of Borman, Stuart & Wheeler (1944) or as *Paracolobactrum coliforme* (Buttiaux, Moriamez & Papavassiliou, 1956).

Group I, type d strains (91 isolates) were anaerogenic organisms which fermented lactose promptly and gave reactions similar to those of type a. They are considered to be anaerogenic variants of *Escherichia* (Report, 1958).

Group I, type e strains (3 isolates) did not ferment lactose at 30°, 37° or 45° in MacConkey's broth. They were nonmotile and remained so after several transfers in the soft agar medium. No gas was produced from glucose in the GA medium on first isolation, but when subcultured in a medium without glucose they later showed gas production in the GA medium. Dissociation into two types of colony, a transparent form with an irregular edge and a more opaque form with an entire edge, was observed on plates of DCA. Independent of the type of colony from which they were isolated, substrains showed prompt, delayed or no gas production from carbohydrates, including glucose, and variations were also observed in ability to ferment other carbohydrates promptly. Mannose, galactose, fructose, rhamnose, xylose, arabinose, maltose, trehalose, mannitol, glycerol, adonitol and dextrin were fermented promptly or with a delay of 3–10 days in the PM medium. Cellobiose, sucrose, dulcitol, inositol, raffinose, salicin and inulin were not fermented at 30° or 37°. The three type e strains were unable to utilize citrate on the modified Christensen medium. This has been described by Edwards & Ewing (1955) and confirmed by Szturm-Rubinsten & Piéchaud (1959) as being a character typical of the *Shigella* group. During this work, however, many of the type a, b and c strains of group I tested were citrate negative on the modified Christensen medium, although their other characters were not typical of *Shigella* strains. All the type d (anaerobic strains) were positive in this test. The combination of biochemical characters exhibited by type e strains, except for carbohydrate fermentation, would indicate that these strains were typical of the *Shigella* group (Report, 1958). However, neither suspensions of living cells nor cells heated at 100° for 2 hr were agglutinated in Sonne, Shiga or Flexner sera obtained from the Institut Pasteur, Paris, and before it can be said with certainty that these strains should be classified as *Escherichia*, their serological group must be determined (Kauffmann, 1959). They have, however, been included as aberrant forms of group I. For the present they should be considered as members of the genus *Paracolobactrum* (Borman, Stuart & Wheeler, 1944) or as an indole negative variant of *Paracolobactrum coliforme* (Buttiaux, Moriamez & Papavassiliou, 1956).

Several group I strains (types a–d) gave weak reactions (pale purple) for indole with the xanthydrol reagent after 2 days and also when retested after incubation for 7 days. This might have been due to the gradual appearance of an adaptive enzyme or to the presence of a mixture of indole positive and indole negative cells in the culture. Several strains of this group which produced indole strongly, weakly or not at all were selected and six successive subcultures made at intervals of

9–15 hr in the tryptone medium, the xanthydrol test being carried out each time after incubation for 2 and 7 days. At the sixth subculture all the strains retained their original character; in particular a weakly positive reaction was still given by the 'weak' strains, an indication that adaptive enzymes had not gradually appeared. Decimal dilutions of cultures in the same medium were plated with nutrient agar and substrains were isolated from the 10^{-9} dilution. Substrains of a 'weak' strain also gave weak reactions with the xanthydrol reagent and there were no changes in the reactions of substrains from indole positive strains. However, 1 substrain out of 17 from an indole negative strain was strongly positive to the test after incubation for 2 days, indicating that indole positive mutants had developed in it.

In all 104 strains of group I (types a–d) were tested for slide agglutination against the *Escherichia* O group sera 26, 55, 86, 111, 119, 125, 126, 127 and 128. Only 2 were positive, with serum 26, and agglutinated to a titre of 1:1,600 in the tube test. All the rest were wholly negative.

Group II. These strains (3 isolates) bore strong resemblances to the *Citrobacter* group (Report, 1958), particularly because of their ability to utilize ammonium citrate, but differed in that H_2S was not produced in the gelatin medium at 22°. Individual strains exhibited other points of variation, namely in the hydrolysis of urea (2 positive) and utilization of malonate (1 positive). Strains of this group have been provisionally named *Citrobacter*-like.

Group III. The 2 strains of this group did not conform to any of the definitions given in Report (1958), and were intermediate between the *Escherichia* and *Cloaca* (*Aerobacter*) groups. They produced NH_3 in the mineral salts-yeast extract-arginine medium under aerobic conditions, but the tests for ornithine and for keto compounds were negative. Strains of this group have been designated 'unclassified type a'.

Group IV. These strains (9 isolates) were nonmotile organisms giving a very mucoid surface growth and producing acid and gas in the GA medium, followed by 'reversal' of the indicator at the surface within 3 days.

Type a strains (6) produced lysine decarboxylase, urease in Hormaeche & Munilla's medium at 37° and gas from insoluble starch, and did not produce phenylalanine deaminase or arginine dihydrolase. These characters justify the inclusion of these organisms in the *Klebsiella* group (Report, 1958; Hormaeche & Edwards, 1958).

Type b strains (3) differed from the others in that they destroyed choline. One of the two indole positive strains produced a slight amount of H_2S in ferrous chloride-gelatin at 22°. The indole positive strains were slightly delayed in lactose fermentation (2 days) and the other strain liquefied gelatin. Slow lactose-fermenting (Henriksen, 1950) and indole positive *Klebsiella* strains have been described (De Issaly, Pennimpede & Issaly, 1954; Ørskov, 1955*a*,*b*, 1957; Edwards & Fife, 1955; Lautrop, 1956; Report, 1958; Hugh, 1959) and although a strong correlation between indole and gelatin liquefaction was demonstrated, gelatin negative and indole positive strains were also observed. The two indole positive strains may be identical to those named *K. oxytoca* (Lautrop, 1956; Ørskov, 1957; Hugh, 1959).

Group V. These strains (29 isolates) were motile organisms giving similar reactions to group IV in the GA medium, but with a nonmucoid surface growth and the reactions shown in Table 2. Some strains fermented lactose slowly and produced 'red' and 'yellow' colonies on DCA plates. These strains also differed from group IV strains in that they were lysine decarboxylase negative, produced arginine dihydrolase, and did not form gas from insoluble starch or show urease production in Hormaeche & Munilla's medium. Hormaeche & Edwards (1958) outlined the controversy concerning the nomenclature of motile organisms related to *Klebsiella* which have been placed in the group *Cloaca* (*Aerobacter*) (Report, 1958). They discussed the propriety of retaining the name *Aerobacter* for the group and defined two species. More recently they have proposed the generic name *Enterobacter*, with the two species *Ent. aerogenes* and *Ent. cloacae* (Hormaeche & Edwards, 1960). Group V strains resembled *Cloaca* (*Aerobacter*) (Report, 1958), and except for the absence of gelatin liquefaction at 22° also resembled *Ent. cloacae* (Hormaeche & Edwards, 1960).

Group VI. Six motile strains resembling group V in colonial appearance on DCA plates and in action in the GA medium were placed in this group. They differed from group V in that no arginine dihydrolase was produced and that Christensen's urease test was negative. They would appear to be related to the *Cloaca* (*Aerobacter*) group but for their inability to produce urease. Since they are both lysine decarboxylase and arginine dihydrolase negative they do not comply with the descriptions for either of the two species given by Hormaeche & Edwards (1960). Lactose nonfermenting organisms resembling the *Cloaca* (*Aerobacter*) group have been placed in the group *Hafnia* (Møller, 1954; Report, 1958). The two lactose negative strains isolated were, however, lysine decarboxylase negative and did not produce H_2S at 22°. Although included in group VI these two strains should be considered for the present as members of the genus *Paracolobactrum* (Borman, Stuart & Wheeler, 1944) or as the species *Paracolobactrum aerogenoides* (Buttiaux, Moriamez & Papavassiliou, 1956). All the strains of group VI have been designated 'unclassified type b'.

Group VII. This group comprised 23 motile. lactose nonfermenting strains producing less acid than groups I to VI in the GA medium with invariably a small volume of gas. The reactions of the various strains composing the group are given in Table 6. They were identified as *Proteus* spp. (Rustigian & Stuart, 1945; Proom & Woiwod, 1951; Cowan, 1956; Ewing, 1958). According to the terminology given by Ewing (1958), type a (17 strains) may be named *Pr. vulgaris*, type b (2 strains) *Pr. mirabilis* and type c (4 strains) *Pr. rettgeri*, although slight differences have been obtained for carbohydrate fermentation. All strains destroyed choline and deaminated phenylalanine and arginine (under aerobic conditions) in mineral salts-yeast extract media with a corresponding positive '2:4-D' reaction for keto compounds. In the case of *Pr. mirabilis*, which gave a negative reaction in the Ehrlich test for indole, the xanthydrol reaction is of particular interest. A distinctive colour reaction (Prussian blue) was obtained in the tryptone and 'combined' media: this indicates the presence of other products of tryptophane breakdown.

Group VIII. These strains (10 isolates) produced the same amount of acid as group VII strains, and a small volume of gas (7 strains) or no gas (3 strains) in the GA medium. They resembled group VII except for their inability to hydrolyze urea. Indole produced during the first day of incubation was afterwards destroyed, and so a weak or negative reaction was observed after incubation for 2 days. Five strains liquefied gelatine very slowly and all the strains produced H_2S in the ferrous chloride-

Table 6. *The classification into types of the 23 group VII* (Proteus) *strains from the alimentary tract of pigs*

Test		Reactions* and no. of strains of		
		Type a (17)	Type b (2)	Type c (4)
Reaction in GA medium		ag	ag	ag (2a)
Indole production	Ehrlich test	+	−	+
	Xanthydrol test	+	−	+
Xanthydrol reaction in tryptone medium		Purple	Prussian blue	Purple
Growth at 45° in MacConkey's broth		−	+	−
Gelatin liquefaction (22°)		+	+	−
V-P reaction		−	+	−
M.R. reaction		+	+	+
Ornithine decarboxylase		−	+	−
Ammonium citrate utilization		+ (6−)	+	+
Citrate utilization (Christensen)		+ (6−)	+	+
Fermentation of	Sucrose	AG	AG†	−
	Maltose	AG (5A, 6−)	−	−
	Mannitol	− (5AG)	−	A (1Ag, 1−)
	Inositol	− (3a)	−	− (2A)
	Dulcitol	−	−	−
	Salicin	− (8AG)	−	AG (2−)
	Adonitol	− (3A)	−	A
Tentative name		*Pr. vulgaris*	*Pr. mirabilis*	*Pr. rettgeri*

* Reactions: +, positive; −, negative; A, acid; G, gas; a, slight acid; g, slight gas. A number before a symbol shows how many strains gave the indicated reaction.

† Slow fermentation.

gelatin medium. Strains of this group are identical with the Providence group (Buttiaux, Frenoy & Moriamez, 1953–54; Proom, 1955; Cowan, 1956; Report, 1958).

In the latter part of the work, small lenticular deep colonies typical of lactose non-fermenting or slow fermenting strains were preferentially isolated from DCA plates (Table 2). In some cases these small colonies were almost indiscernible with the naked eye and so may often have been overlooked, or they may have been hidden by large neighbouring colonies. When there was a risk of overgrowth by large colonies the small colonies were isolated after 24 hr; but if there was no such risk the plates were incubated for a further 24 hr to allow maximum development. Two groups of bacteria other than Enterobacteriaceae were identified during this part of the investigation. Descriptions of these now follow.

(ii) *Brucellaceae*

Group IX. Type a strains (15 isolates) were from the following sources: 3 strains from the faeces of a control animal; 11 strains from the caecum of an animal receiving an aureomycin supplement; and 1 strain from the faeces of an animal receiving a neomycin supplement. Type b strains (16 isolates) were from the following sources: 11 strains from the faeces of 3 control animals; and 5 strains from the faeces of an animal receiving an aureomycin supplement.

Surface colonies on DCA plates of both type a and b strains were small (1–2 mm diam.), yellow and transparent. Deep colonies were small, red and lenticular. Cultures on nutrient agar slopes were composed of small, often coccoid rods in chains, groups and filaments. They were motile at 22° (but not at 30° or 37°) by means of peritrichous or subterminal flagella (Plate 1a). This latter character is typical of *Pasteurella pseudotuberculosis* (Breed, Murray & Smith, 1957).

Table 7. *The biochemical behaviour of group IX* (Pasteurella pseudotuberculosis) *strains from the alimentary tract of pigs*

Test	Reactions* and no. of strains of	
	Type a (17†)	Type b (16)
Reaction in GA medium at 22°	a	agr‡
Reaction in GA medium at 30°	a	a
Reaction in GA medium at 37°	a	a
Gas in nutrient agar at 22°	–	+
Gas in nutrient agar at 30°	–	(+)
Gas in nutrient agar at 37°	–	–
Lactose fermentation	–	–
Indole production	–	–
Lysine decarboxylase	–	–
Growth in MacConkey's broth at 45°	–	–
$FeCl_2$–gelatin medium (22°): Gelatin liquefaction	–	–
$FeCl_2$–gelatin medium (22°): H_2S production	–	–
Urease (Christensen)	+	+
Urease (Hormaeche & Munilla) (37°)	+	+
V-P/M.R. reactions at 22°	–/+	+/(+)‡
V-P/M.R. reactions at 30°	–/+	–/(+)
V-P/M.R. reactions at 37°	–/+	–/(+)
Phenylalanine deaminase	–	–
NH_3 from arginine§	–	–
'2:4-D' reaction§	–	–
Arginine dihydrolase	–	–
Arginine decarboxylase	–	–
Ornithine decarboxylase	–	+
Malonate utilization	–	–
Ammonium citrate utilization	–	–
Citrate utilization (Christensen)	–(1+, 5 days)	–(1+, 5 days)
Motility at 22°	+	+
Motility at 30°	–	–
Motility at 37°	–	–
Nitrate reduction	+	+
Cytochrome oxidase	–	–
Fermentation of Mannose	A (2–)§§	A (8–)
Galactose	A	A
Fructose	A	A
Rhamnose	A	–
Xylose	A	–
Arabinose	A (1–)§§	A
Maltose	A	A**
Cellobiose	–	A
Sucrose	–	A
Trehalose	A	A
Mannitol	A	A
Dulcitol	–	–
Inositol	–	A(2–10 days)
Glycerol	A***	A****
Raffinose	–	–
Adonitol	–	–
Salicin	a	–
Inulin	–	–
Dextrin	A	A
Aesculin hydrolysis	+	–

* Reactions: +, positive; –, negative; (+), slight reaction. or orange red colour in the M.R. test; A, acid; a, slight acid; g, small bubble of gas at surface of GA medium; r, 'reversal' of indicator to neutral at the surface of GA medium; A number before a symbol shows how many strains gave the indicated reaction.

† Includes 1 strain from the liver of a cat received from Dr. Piéchaud and 1 strain from the collection of the Institut Pasteur, Paris.

‡ One strain gave no reversal on GA medium and was V-P negative. § Arginine medium AM.

§§ This strain was a colonial variant that developed after subculture on DCA.

** Seven strains produced acid only after 2–3 days.

*** Three strains produced acid only after 2–5 days.

**** One strain produced acid only after 4 days.

It may be seen from Table 7 that type a strains were also typical of *Past. pseudotuberculosis* in biochemical behaviour (Breed *et al.* 1957). The media and methods employed were the same as those described for Enterobacteriaceae strains and any differences in the temperature of incubation are noted in Table 7. The PW medium was used for carbohydrate fermentation. Table 7 shows that type b differed from type a strains in the ability of the former to produce gas in the nutrient agar medium at 22° and 30° and acetoin from glucose at 22°. A slight bubble of gas was also produced by type b strains in the PW medium at 22° and 30° whether a fermentable carbohydrate was present or not. Type b strains also differed in that ornithine was decarboxylated and mannose, rhamnose, xylose, salicin and aesculin were not attacked, whereas cellobiose and sucrose were fermented. Two type a strains were unable to ferment mannose. One of these was a colonial variant which arose on artificial culture and was also unable to ferment arabinose.

Intraperitoneal injection of 0·5 ml of a 24 hr broth culture of a type a strain into a guinea pig had no ill effect on the animal. After the animal was killed two months later, post mortem examination revealed a normal appearance of the internal organs. Intraperitoneal injection of the same amount of a culture of a type b strain had no serious effect on a guinea pig, but a superficial purulent abscess formed at the site of the injection several days later. The organism inoculated was cultivated from the pus. When the animal was killed two months later the abscess was still present, but the internal organs were normal in appearance. Two other guinea pigs were inoculated intraperitoneally, one with a strain of *Past. pseudotuberculosis* received from the collection of the Institut Pasteur, Paris, and the other with a strain received from Dr. Piéchaud: this he had isolated from the liver of a cat. These 2 strains killed the animals within 3 weeks and lesions typical of pseudotuberculosis were found at post mortem. Pure cultures of the organism were isolated from the lesions.

Strains of type a were lyzed by phage specific for the genus *Pasteurella* (received from the Institut Pasteur). Type b strains showed normal growth in the presence of this phage. Type a strains agglutinated with *Past. pseudotuberculosis* group 3 antiserum (received from Dr. Mollaret), but type b strains were autoagglutinable.

The cultural and biochemical characters, including carbohydrate fermentations, of type a strains agree with the descriptions given for *Past. pseudotuberculosis* by Zlatogoroff & Moghilewskaja (1928), Schütze (1928), Brigham & Rettger (1935), Fauconnier (1950), Piéchaud (1952), Devignat & Boivin (1954), Thal & Chen (1955) and Breed *et al.* (1957) and with the results obtained from the 2 strains received from outside sources. Although type b strains differed from the accepted description of the species in certain biochemical characters, including carbohydrate fermentations, their cultural characters and morphology suggested that they be considered as atypical strains of *Past. pseudotuberculosis.*

Group X. This comprised 20 strains, all of which were isolated from pinpoint to small (1 mm diam.) deep colonies on DCA plates. Type a strains (16 isolates) were from the following sources: 14 from the caecal contents of 4 control animals receiving either ration A or C and 2 from the faeces of 2 animals (1 receiving ration A and 1 receiving an aureomycin supplement). Type b strains (4 isolates) were isolated at the same time as type a from the caecal contents of a control animal receiving ration C. Reinoculation from an 18 hr LAPTg5 culture on to the surface of DCA resulted in the growth, after 48 hr, of scattered pinpoint or larger (1–2 mm diam.) slightly raised, translucent, red or yellow colonies with entire edges. When touched with a platinum wire the whole colony could be pulled away. Growth occurred on nutrient agar slopes as small colonies (1–2 mm diam.), but it was difficult, if not impossible, to subculture directly from one agar slope to another. Pleomorphic small fine rods ($0{\cdot}5\mu\times4\mu$) and coccoid forms ($0{\cdot}8\mu\times1\mu$) were observed on this medium; these were often in pairs, chains of 3–4 and masses. In LAPTg5 small coccoid rods predominated; these were often in chains or radially arranged in masses (Plate 1b). There was a suggestion of capsules on first isolation. The organisms were not motile at 37°, 30° or 22° and growth at 22° was slow. Strains were viable for only 3–4 days at room temperature in liquid or solid media. Growth on mineral salts-yeast extract media was poor and no growth occurred on potato slopes. Slight growth occurred on the slope of coagulated serum and there were no floccules in the syneresis fluid. Growth was more abundant on Löffler's coagulated serum and microscopical examination of an unstained mount from the slope revealed tiny refringent coccoid forms. There was no liquefaction of coagulated serum or of gelatin and growth in the latter medium was poor. Small colonies, which sometimes reached a diameter of 2 mm, developed on Brucella agar and discrete white colonies (1–2 mm diam.) developed on sheep's blood agar, without producing haemolysis.

These morphological and cultural characters are typical of members of the genus *Actinobacillus* (Breed *et al.* 1957).

Table 8. *Some biochemical characters of strains of group X* (Actinobacillus) *from the alimentary tract of pigs and of strains of* A. lignieresii *and* A. equuli

Tests	Reactions* and no. of strains of			
	Group X, type		*A. lignieresii*†	*A. equuli*‡
	a(16)	b(4)	(6)	(3)
Reaction in GA medium	(a)	(a)	(a)	(a)
Lactose fermentation	A (1–2 days)	—	a (7 days)	—
Indole formation	—	—	—	—
Lysine decarboxylase	—	+ (2 days)	—	—
Growth in MacConkey's broth at 45°	—	—	—	—
Gelatin liquefaction**	—	—	—	—
Choline production**	—	—	—	—
Urea destruction**	+	+	+	+
Indole production**	—	—	—	—
Gelatin liquefaction***	—	—	—	—
H_2S production***	—	—	—	—
Urease (Christensen)	+	+	+	+
Urease (Hormaeche & Munilla) (37°)	—	+	—	—
V-P/M.R. reactions	—/+	(+)/+	(+)/+ §§	—/(+)
Phenylalanine deaminase	—	—	—	—
NH_3 from arginine§	—	—	—	—
Arginine dihydrolase	—	—	—	—
Arginine decarboxylase	—	—	—	—
Ornithine decarboxylase	—	+	—	—
Malonate utilization	—	—	—	—
Ammonium citrate utilization	—	—	—	—
Citrate utilization (Christensen)	+	—	—	—
Nitrate reduction	+	+	+	+
Cytochrome oxidase	—	—	—	—
Motility††	—	—	—	—
Action on litmus milk	aG	—	—	—
Catalase	—	+	+	+

* Reactions: +, positive; —, negative; (+), slight; A, acid; a, slight acid; (a), very slight acid; G, gas in 24 hr; † Strains received from Dr. Phillips. ‡ Strains received from Dr. Vallée.

** Tests done in 'combined' medium at 37°. *** Tests done in $FeCl_2$-gelatin medium at 22°. § Arginine medium AM. †† Tests done on cultures grown at 37°, 30° and 22°

§§ One strain was V-P and M.R. negative.

Subcutaneous injection of 0·5 ml of an 18 hr LAPTg5 culture into a guinea pig resulted in the formation of a subcutaneous induration after 24 hr. The animal appeared ill and did not eat. The indurated area gradually disappeared and the animal was normal after 4 days. At autopsy 3 months later a normal appearance of the internal organs was revealed.

The biochemical behaviour of the group X strains types a and b is shown in Table 8, together with the results obtained under the same conditions for strains of *Actinobacillus lignieresii* (Plate 1c) from actinobacillosis lesions in cattle and sheep (received from Dr. Phillips) and strains of *A. equuli* (Plate 1d) isolated by Dr. Vallée from foals. The media and methods employed were the same as those described for Enterobacteriaceae, but except for lactose fermentation and indole production incubation was at 37° instead of 30°. The results of carbohydrate fermentations for these strains are given in Table 9. It may be seen from Tables 8 and 9 that although many characters were common to all strains there was an important difference in that carbohydrates were fermented in the LAPT basal medium with gas production by group X strains, whereas the strains of *Actinobacillus* received from outside sources produced acid only.

Since group X strains closely resembled the various species of *Actinobacillus* in morphology, cultural characteristics and pathogenicity, a review of the literature was undertaken with the purpose of identifying them more fully.

Four species were described by Breed *et al.* (1957): *A. lignieresii*, *A. actinomycetemcomitans*, *A. equuli* and *A. actinoides*. These names will be employed for the following review, although others may have been used by the authors cited. Descriptions of the morphological and cultural characteristics given by different workers agree with each other and also correlate with those described for group X strains. There is a close resemblance between the first three species listed above, although *A. equuli* may be distinguished by its viscous nature in culture. The fourth species contains the organism first isolated by Smith (1918, 1921*a*,*b*) from pneumonic lungs of calves. A similar organism was later isolated from a case of pneumonia in the rat by Jones (1922). These workers distinguished the species by the production of floccules in the syneresis fluid of coagulated blood serum media. On microscopical examination these floccules were shown to contain club shaped organisms.

Reports have varied with regard to the biochemical behaviour of different strains within the first three species. No description was given for the biochemical characters of the fourth species. *A. lignieresii* strains were described by Breed *et al.* (1957) as capable of forming indole. Lignières & Spitz (1902, 1903) and Thompson (1933*b*) obtained a weak indole reaction with strains from cattle after 4–5 days, and Beaver & Thompson (1933) found that a strain from man was positive after 3 days; but other workers have reported negative results (Bosworth, 1923; Magnusson, 1929; Davies & Torrance, 1930; Tunnicliff, 1941; Taylor, 1944; Ristic, Herzberg, Sanders & Williams, 1956; Phillips, 1960). Haupt (1934) considered *A. actinomycetemcomitans* strains to be identical with *A. lignieresii* and distinguished these species from *A. equuli* on the basis of the reduction of nitrate to nitrite by the latter. However, other workers who have carried out this test (Tunnicliff, 1941; Phillips, 1960) have obtained a positive result for *A. lignieresii*. The following results have been obtained for *A. lignieresii* strains in litmus milk: definitely acid but never coagulated (Lignières & Spitz, 1902, 1903); no change or coagulation (Magnusson, 1929); no change (Vawter, 1933; Taylor, 1944); no change or acid formation (Jowett, 1931); slight reduction (Wramby, 1940); slight acidity or little or no change (Davies & Torrance, 1930); and acid production (Bosworth, 1923). *A. actinomycetemcomitans* strains isolated by Vallée & Gaillard (1953) produced no change in litmus milk, whereas no growth was reported in this medium by a strain isolated by Thjøtta & Sydnes (1951). Cultures of *A. equuli* in litmus milk became very viscid and slimy and slowly acid (Snyder, 1924). It may be seen from Table 9 that considerable variations in carbohydrate fermentation have been obtained for *A. lignieresii* strains. Nevertheless, the results of the majority of workers show that strains of this species from various sources formed acid (often slight) from the following sugars: glucose, mannose, galactose, fructose, xylose, maltose, sucrose, mannitol and lactose (often slowly fermented). A high proportion of lactose, raffinose, glycerol and dextrin negative strains have been isolated. Arabinose, rhamnose and salicin were generally negative, and the results show that dulcitol, inositol, adonitol and inulin were not fermented by strains of this species. Except in the case of maltose and mannitol *A. actinomycetemcomitans* strains gave the same results for the few carbohydrate fermentation tests reported for this species. There are slight variations in the results reported for *A. equuli* strains, particularly for xylose, glycerol, arabinose, dextrin, salicin, adonitol and inulin fermentation. This ability of certain strains of *A. equuli* to ferment adonitol and inulin constitutes a difference in biochemical behaviour between them and *A. lignieresii*. In recent publications (Ristic *et al.* 1956; Phillips, 1960), the test for a catalase enzyme has been employed for *A. lignieresii* strains. All strains studied by Ristic *et al.* were positive, whereas of the 225 strains studied by Phillips 213 were positive and 12 negative. He also reported that 82 strains were urease positive and 143 were negative.

Strains of *Actinobacillus* spp. have been reported as having a variable pathogenicity for guinea pigs. Tunnicliff (1941) stated that, in his experience, strains of *A. lignieresii* from sheep gradually lost their virulence in artificial culture although they killed inoculated guinea pigs when first isolated. An intraperitoneal inoculation of a bovine strain by Bosworth (1923) resulted in the guinea pig becoming "ill, dull and dejected" within 24 hr, with the abdomen "tense and painful". The following day the animal was "brighter" and very quickly recovered to normal. One strain, however, had a greater virulence and a guinea pig died with a general peritonitis 16 hr after inoculation, whereas a second animal survived for 14 days. There had been a swelling of the testicles which had subsided on the 6th day and at post mortem there were lesions in the abdominal cavity and nodules containing pus on the peritoneal covering of the liver. A pure culture of the organism inoculated was obtained from the pus which, on microscopical examination, revealed club formation typical of actinobacillosis. A strain of *A. lignieresii* isolated from man (Beaver & Thompson, 1933) was pathogenic for guinea pigs, whereas Thompson & Willius (1932) reported that a guinea pig inoculated intraperitoneally with another human strain was ill for 2 days but recovered in 3–4 days. Davies & Torrance (1930) found their bovine strains nonpathogenic for guinea pigs on intraperitoneal injection of a whole agar culture

Table 9. *A comparison between published carbohydrate fermentation reactions and those of isolates and named strains of the genus* Actinobacillus

Strain, origin and reference*	No. of strains	Result† of fermentation of: Glucose	Mannose	Galactose	Fructose	Xylose	Maltose	Sucrose	Mannitol	Lactose	Raffinose
A. lignieresii											
Cattle[1]	?	A								A	
Cattle[2]	17	A		A	A		A	A		A	
Cattle[3]	1					A	A	A	A	a (4–14)	a (4–14)
Sheep[4]	2	A	A	A	A	A	A	A	A	a (6)	—
Cattle[5]	28	A		A	A	A	A	A	A	A	
Sheep[6]	?	A				A	A	A	A	A (6)	
Man[7]	?	a					a	a	a		
Man[8]	1	a					—	a	a	—	
	1	A					A	—	A	—	
Cattle[9]	15	A					A	A	A	A	
Sheep[10]	18‡	A		A	A	a	A	A	A	A (4–10)	A (4–10)
?[11]	4	A		A	A	A	A	A	A	—	−3, A1
Cattle[12]	?	A			A	A	A	A		A	
Sheep[13]	?	A	A	A	A	A	A	A	A	A	—
Sheep[14]	2	A1, −1	A	A	A	A	A	A	A	A	—
Cattle[14]	5	A4, a1	A	a2, A1, −2	A	A	A	A	A	A4, −1	−3, A1, a1
Man[14]	1	A	A	A	A	A	—	—	A	—	—
Sheep[15]	7	A	A	A	A	A	A	A	A	A	A or —
Cattle and sheep[16]	13	A	A	A	A12, −1	A	A	A	A	−6, A6, A1§	—
ATCC[16]§§	1	A	—	A	—	—	A	—	—	—	—
Cattle and sheep[17]	225	A	A	A127, A98 (2–14)	A	A	A	A222 A3, (2–5)	A	A87, −38	−175, A50 (4)
A. actinomycetem-comitans											
Man[18]	1	A						A	A	A	
Man[19]	?	A								A	
Man[20]	1	a		a			—	a	—	a	
White mice[21]	?	A					A		A		
A. equuli											
Foal[22]	?	A		A			A	A	A	A	A
Foal[23]	?	A			A	A	A	A	A	A	
Foal[24]	40	A		A	A	A	A	A	A	A	A
Foal[25]	6	A		A	A	A5, −1	A	A	A	A	A
Group X, type a** Pig (caecum, faeces)	16	AG	AG	AG	AG	AG	AG	AG	—	AG	AG (2–4)
Group X, type b** Pig (caecum)	4	AG	AG	AG	AG	AG	AG	AG	—	—	—
A. lignieresii†† Cattle and sheep	6	a	a	a	a	a	a	a	a	a (7)	—
A. equuli††† Foal	3	a	a	a	A	A	a	a	−2, a1	a	a

* References, [1], Lignières & Spitz (1903); [2], Bosworth (1923); [3], Magnusson (1928); [4], Magnusson (1929); [5], Davies & Torrance (1930); [6], Jowett (1931); [7], Thompson & Willius (1932); [8], Beaver & Thompson (1933); [9], Thompson (1933*a*,*b*); [10], Vawter (1933); [11], Haupt (1934); [12], Davis & Stiles (1939); [13], Wramby (1940) cited by Taylor (1944); [14], Tunnicliff (1941); [15], Taylor (1944); [16], Ristic *et al.* (1956); [17], Phillips (1960); [18], Colebrook (1920); [19], Bayne-Jones (1925); [20], Thjøtta & Sydnes (1951); [21], Vallée & Gaillard (1953); [22], Magnusson (1919); [23], Snyder (1924); [24], Edwards (1931); [25], Haupt (1934).

† Result: A, acid; AG, acid and gas; a, slight acid; —, negative. The numbers not in brackets are those of strains giving the indicated reaction and the

named strains of the genus Actinobacillus

Strain, origin and reference*	No. of strains	Result† of fermentation of										
		Glycerol	Arabinose	Rhamnose	Dextrin	Salicin	Trehalose	Dulcitol	Adonitol	Inulin	Inositol	Cellobiose
A. lignieresii												
Cattle[1]	?											
Cattle[2]	17											
Cattle[3]	1		—	—		—		—	—	—		
Sheep[4]	2	—	—	—		—		—	—	—	—	
Cattle[5]	28					—		—	—	—	—	
Sheep[6]	?		—	—		—		—	—			
Man[7]	?											
Man[8]	1					—		—		—		
	1					A		—		—		
Cattle[9]	15					—		—		—		
Sheep[10]	18‡	A (14–21)	—			—		—		—		
?[11]	4	—	—	—	A	A		—	—	—		
Cattle[12]	?											
Sheep[13]	?	A	—	—	A	—	—	—	—	—	—	
Sheep[14]	2	A1, —1	—	—	—	—	—	—				
Cattle[11]	5	—4, A1	—	—	—	—	—	—				
Man[14]	1	—	A	A	—	—	—	—				
Sheep[15]	7	A or —	A or —	A	—	—	—		—	—	—	
Cattle and sheep[16]	13	—	—10, A1, A2§	—	A6, —4, A3§	—	—	—		—		
ATCC[16]§§	1	—	—	—	A	—	—	—		—		
Cattle and sheep[17]		A163 (5), —62	A197 (7–14), —28	—224, A1	A	—	—	—		—	—	
A. actinomycetemcomitans												
Man[18]	1				A			A				
Man[19]	?											
Man[20]	1	—										
White mice[21]	?		A									
A. equuli												
Foal[22]	?		—	—				—	—			
Foal[23]	?		—	—	—	A		—				
Foal[24]	40	A34 (slow), —6	—35, A5	—	A37, —3	—36, A4		—	—32, A8 (slow)	—38, A2		
Foal[25]	6	—5, A1	A3, —3	—	—	—4, A2		—	—	—5, A1		
Group X, type a** Pig (caecum, faeces)	16	a (2–4)	AG	—9, AG7	AG	—	AG	—	—	—	—14, AG2	—
Group X, type b** Pig (caecum)	4	a	AG	—	AG	—	—	—	—	—	—	—
A. lignieresii†† Cattle and sheep	6	a (3–4)	—	—	a	—	—	—	—	—	—	—
A. equuli††† Foal	3	—	A2, —1	—	a	a2, —1	a	—	—	—	—	a2 (8–14), —1

* References, [1], Lignières & Spitz (1903); [2], Bosworth (1923); [3], Magnusson (1928); [4], Magnusson (1929); [5], Davies & Torrance (1930); [6], Jowett (1931); [7], Thompson & Willius (1932); [8], Beaver & Thompson (1933); [9], Thompson (1933*a*,*b*); [10], Vawter (1933); [11], Haupt (1934); [12], Davis & Stiles (1939); [13], Wramby (1940) cited by Taylor (1944); [14], Tunnicliff (1941); [15], Taylor (1944); [16], Ristic *et al.* (1956); [17], Phillips (1960); [18], Colebrook (1920); [19], Bayne-Jones (1925); [20], Thjøtta & Sydnes (1951); [21], Vallée & Gaillard (1953); [22], Magnusson (1919); [23], Snyder (1924); [24], Edwards (1931); [25], Haupt (1934).

† Result: A, Acid: AG, acid and gas; a, slight acid; — negative. The numbers not in brackets are those of strains giving the indicated reaction and the numbers in brackets are the days taken for the reaction to appear.

‡ Including one strain from Magnusson. § Acid produced at 30° but not at 37°. ** Tested in LAPT. §§ ATCC, American Type Culture Collection. †† From Dr. Phillips; ††† From Dr. Vallée.

suspension. Guinea pigs were not susceptible to the ovine strains studied by Magnusson (1929) (irrespective of the mode of injection used) and by Jowett (1931). Two guinea pigs inoculated with *A. actinomycetemcomitans* by Colebrook (1920) died with diffuse peritonitis and a third died within 30 days with congestion of the *tunica vaginalis*, but no peritonitis. A subcutaneous injection produced an indurated inflammatory reaction but no abscess. Strains of *A. equuli* were found by Magnusson (1929) not to be pathogenic irrespective of the method used for injection. A similar result was obtained by Snyder (1924) after subcutaneous and intraperitoneal injections.

Although this review has not been exhaustive it is apparent that reports differ with respect to the biochemical behaviour and pathogenicity of strains within the various species of *Actinobacillus*, and that the demarcation between them is ill defined.

In no case have the biochemical characteristics described for *Actinobacillus* spp. agreed exactly with those of group X strains. The essential differences are the range of carbohydrates fermented and the ability of group X strains to produce gas. When the basal medium has been described in reports it has been a peptone-meat extract or an infusion medium. In the PW and PM media containing fermentable carbohydrate, group X strains produced only acid at 30°, although in some cases a small bubble of gas (which could easily have been overlooked) was formed. A slight amount of gas was formed in these media at 37° from fermentable carbohydrates and a large amount of gas was formed from the same carbohydrates (except glycerol) in the LAPT basal medium at this temperature. However, 6 strains of *A. lignieresii* of bovine and ovine origin received from Dr. Phillips and 3 strains of equine origin received from Dr. Vallée did not produce gas from fermentable carbohydrates under any of these conditions. Inability of group X strains to ferment mannitol constitutes another important difference, although two mannitol negative strains of the genus *Actinobacillus* have been reported in the literature. One, from the American Type Culture Collection (Ristic *et al.* 1956), which was named *A. lignieresii*, differed from group X strains also in not fermenting mannose, fructose, xylose and sucrose; and the other, designated *A. actinomycetemcomitans* (Thjøtta & Sydnes, 1951), which was isolated from an *Actinobacillus* infection in a young woman, differed from group X strains in that maltose and glycerol were not fermented. Two of the strains of *A. equuli* received from Dr. Vallée were also unable to ferment mannitol. However, they differed from group X strains, since both fermented salicin promptly and cellobiose slowly, and one did not ferment arabinose.

Group X strains have been isolated from apparently healthy pigs, whereas *Actinobacillus* spp. have been isolated only from cases of disease in animals and man. The majority of cases have been of cattle and sheep suffering from a disease of the soft tissues (Magnusson, 1928; Bosworth, 1959) superficially resembling actinomycosis. Cases of actinomycosis of the soft tissues in pigs have been shown to be caused by a Gram-positive organism (Creech, 1927; Magnusson, 1928; Davies & Torrance, 1939). Only one case has been reported in the literature of an *Actinobacillus* infection in pigs (Clarenburg, 1924). The organism was recovered from various organs, including the heart, of a 16 day-old piglet that had died of an acute febrile disease lasting 24 hr. The author considered the organism he isolated to be identical with those Magnusson (1919) had recovered from joint-ill in foals (*A. equuli*). The descriptions of morphological and cultural characteristics comply with those for the genus *Actinobacillus.* Acid without gas was formed from glucose and lactose in one to two days, Ehrlich's test for indole was negative in broth culture after incubation for 3–4 weeks, and neither gelatin nor coagulated blood serum was liquefied; acid was formed in one day from litmus milk and the milk became slimy and viscid in consistency and coagulated on the fourth day. Subcutaneous inoculation of a guinea pig resulted in a painful, hot swelling, with enlargement of the ganglions near the site of the injection; in 4 days, however, the guinea pig had recovered.

The authors consider that there are sufficient points of resemblance to allow the inclusion of group X strains in the genus *Actinobacillus.* However, these strains cannot be given a species designation since no strain of a recognized species has shown the same biochemical behaviour or fermentation pattern, and no strain has been described as producing gas from fermentable carbohydrates.

(c) *Counts obtained on deoxycholate agar plates from different parts of the alimentary canal and from faeces*

Table 10 gives the counts (maximum, minimum and average) obtained on DCA over a period of 4 years from 573 samples of gastric, intestinal and caecal contents and faeces. It shows that Gram-negative facultative anaerobes, among which Enterobacteriaceae were shown to be dominant, were normally present in the alimentary canal and faeces of animals receiving standard diets for growing and fattening, and developing normally. The average numbers (/g of fresh material) were low in the stomach ($4{\cdot}3\times10^4$), slightly higher in the small intestine ($1{\cdot}5\times10^6$) and even higher in the caecum ($6{\cdot}5\times10^6$). Although the highest numbers occurred in the faeces ($1{\cdot}7\times10^7$), they reflected those in the caecum. The maximum and minimum values show that wide variations may be obtained in different samples from the various parts of the alimentary tract studied and from the faeces (which may or may not have been taken from the same animal).

Table 10. *A comparison of counts obtained in deoxycholate agar with samples from the alimentary tract of pigs*

Source of sample	No. of samples	Log_{10} no. of bacteria/g of fresh material		
		Average	Maximum	Minimum
Stomach	125	4·63	6·70	1·00
Small intestine	79	6·17	7·70	3·00
Caecum	103	6·81	8·70	4·70
Faeces	266	7·23	9·20	4·00

(d) *Comparison of deoxycholate agar with other media for the selective enumeration of Gram-negative bacteria in faeces*

This experiment was carried out in order to check that DCA was a suitable medium for enumerating Enterobacteriaceae. Decimal dilutions of faecal samples were plated with violet red-bile agar (VRBA) (Difco), SS agar (Difco) and urea agar (prepared as described in Manual, 1953). The average counts (/g of fresh material) obtained for 12 samples of faeces were as follows: DCA, $1{\cdot}8\times10^7$; VRBA, $1{\cdot}9\times10^7$; SS, $1{\cdot}1\times10^6$; urea agar, $2{\cdot}2\times10^7$.

The VRBA counts corresponded well with those on DCA whereas on SS agar the counts were a tenth of those on DCA. However, isolates from colonies on the highest dilution plates were in all cases typical *Escherichia* strains. The urea agar yielded a higher count, but urease positive colonies and other small colonies which grew on the highest dilution plates were Gram-positive cocci and rods. No *Proteus* spp. were detected in the dominant flora during the 12 examinations made.

4. Discussion

The use of deoxycholate agar for the enumeration of Enterobacteriaceae and other Gram-negative bacteria in the alimentary tract of pigs

The average counts obtained on DCA plates from the contents of various parts of the alimentary canal and from faeces were lower than those reported by Horvath *et al.* (1958), who used dilution methods in MacConkey's broth for the enumeration of 'coliform' organisms. However, the counts for caecal contents and faeces correlate with those obtained by Willingale & Briggs (1955) and Fuller *et al.* (1960), who used methods similar to those of Horvath *et al.* (1958). During the present investigation the counts on DCA were higher than those on SS agar (a more highly selective medium) and lower than those on urea agar (where Gram-positive organisms appeared on high-dilution plates) although comparable results were obtained with DCA and VRBA. Other workers who used solid media selective for 'coliform' organisms have obtained results similar to those reported here. The only discrepancy that has occurred is in the results obtained by Bridges *et al.* (1953). These workers used SS agar, urea agar and VRBA as presumptive media for *Shigella* strains, *Proteus* spp. and 'coliform' organisms, respectively, and obtained higher counts for the two former groups than for the latter. The length of time that had elapsed between sampling and bacteriological examination was not mentioned in their publication; but in a previous paper (Bridges, Dyar & Burkhart, 1952), they mentioned that examinations were made in the 48 hr following sampling. This would perhaps account for the high counts obtained on SS agar and urea agar, as holding the samples may have enabled certain bacteria to multiply that were later able to develop on these media.

It must be stressed that Gram-negative strict aerobes are able to grow on DCA. Colonies typical of these organisms may be recognized on plates prepared from gastric contents in numbers exceeding those typical of facultative anaerobes. At the beginning of the investigation they were also noticed on high dilution plates prepared from faeces which had not been collected immediately after emission. Fresh faeces must therefore be examined and only colonies typical of facultative anaerobes must be counted. When these conditions have been fulfilled the medium appears to be suitable for the selective enumeration of Enterobacteriaceae in the contents of the alimentary tract and faeces of the pig; and, because it is a solid medium, isolations may be made from high dilution plates on which other Gram-negative facultative anaerobes may also be observed.

Modified methods for differentiation of the groups

There was great variation in the morphology and biochemical behaviour of the Gram-negative facultatively anaerobic bacteria isolated, by means of deoxycholate agar, from the alimentary tract and faeces of pigs during this investigation. Members of the Enterobacteriaceae and Brucellaceae have been recognized and these may be divided into 10 groups, each group embracing several biochemical types. This division into groups is arbitrary, as it is based on tests which are only valid under defined experimental conditions. It is therefore important to justify the modifications that have been suggested for some of the tests employed.

Methods for the detection of products of glucose metabolism

The Voges-Proskauer (V-P) reaction for acetoin production. Since a synthetic medium has been shown to be superior to a peptone medium in obtaining an inverse correlation between the M.R. and V-P reactions (Fouad & Richards, 1953; Dickinson, 1956) a mineral salts medium containing glutamic acid and yeast extract was used. A modification of the reagent of Vaughn, Wedding & Tabachnick (1948) was used in the V-P test and in all cases a positive V-P test with this medium corresponded with a negative M.R. reaction and with 'reversal' of the indicator at the surface of the GA medium for *Klebsiella* (group IV), *Cloaca* (*Aerobacter*) (group V), 'unclassified type b' (group VI) and the majority of atypical *Past. pseudotuberculosis* (group IX, type b) strains. Conversely, a negative V-P test was obtained with a positive M.R. test and there was no 'reversal' of the indicator for strains of the other groups recognized. However, with certain *Proteus* (group VII, type b) and *Actinobacillus* (group X, type b) strains an absence of 'reversal' of the indicator was associated with an indefinite M.R. test, and only a slight positive V-P reaction was obtained. This might be explained by the fact that in the GA and V-P media the acidity was not low enough to allow acetoin to be formed, since the optimum pH of the enzyme for acetoin formation is between 5·6 and 6·0 (Silverman & Werkman, 1941).

The glucose agar medium. This has already been shown to be useful for the differentiation of organisms of the 'coliform' groups from one another and from aerobic organisms (Dickinson, 1956) and is similar to the medium described by Hugh & Leifson (1953) for the detection of fermentative *versus* oxidative metabolism by Gram-negative bacteria. The results of the present work confirm the usefulness of the glucose agar medium, particularly as a test in the primary differentiation of groups of Enterobacteriaceae and for the distinction of members of this family from other Gram-negative facultative anaerobes. It is important to note that Bacto peptone must be used in the medium, otherwise the correlation between acetoin production and indicator 'reversal' at the surface is not obtained.

Methods for the detection of products of amino acid metabolism

Attempts have been made to modify the methods so that tests could be carried out on cultures grown in mineral salts-yeast extract media containing the substrate amino acid as the main organic source of nitrogen.

Phenylalanine. A colorimetric method has been described (Friedemann, 1957) for the assay of keto compounds, which depends on the formation of a red colour in excess alkali by the corresponding hydrazones after reaction with 2:4-dinitrophenylhydrazine. This method has been modified for bacteriological use in the present work (the '2:4-D' test) and has been employed in routine examinations for phenylalanine oxidative deaminase by strains grown in a modification of the medium described by Shaw & Clarke (1955). The results have in all cases agreed with a parallel ferric chloride test described by these workers, and only *Proteus* spp. (group VII) and Providence strains (group VIII) have given positive reactions. Although the '2:4-D' test is not specific for a single keto compound, it has the advantage that keto acids other than those which may be distinguished by colour reactions with ferric ions (Henriksen & Closs, 1938; Shaw & Clarke, 1955; Singer & Volcani, 1955; Ben Hamida & Le Minor, 1956; Thibault & Le Minor, 1957) may also be detected.

Arginine. When strains were grown in the arginine medium (AM) only *Proteus* and Providence strains gave positive results in the '2:4-D' and NH_3 tests. This combination of results indicates oxidative deamination of the amino acid.

In a summary of work on the arginine dihydrolase complex, Oginsky (1955) has shown that breakdown of arginine results in the production of ornithine (through citrulline) and NH_3. Møller (1955) determined the reaction products derived from arginine by several members of the Enterobacteriaceae and described a peptone-meat extract medium containing pyridoxal and glucose for arginine decarboxylase and dihydrolase production, in which the presence of NH_3 indicated the action of arginine dihydrolase. Pyridoxal was omitted from the medium in a modification of the arginine dihydrolase test by Hormaeche & Munilla (1957). During the present investigation, the ninhydrin reagent described by Chinard (1952) has been adapted for the detection of ornithine in cultures grown in the AM medium, and a simultaneous test for NH_3 has also been carried out. *Cloaca* (*Aerobacter*) (group V) were positive to the arginine dihydrolase test of Hormaeche & Munilla and both ornithine and NH_3 were produced in the AM medium. The reaction for NH_3 was more definite in some cases and its presence was corroborated by a positive reaction for ornithine. This test for arginine dihydrolase may, therefore, have a slight advantage over previous tests.

Tryptophane. Xanthydrol was shown by Fearon (1944) to give a colour reaction with indole in an acetic acid solution. A modification of this method has been used for the detection of indole production in both the tryptone and combined media. Parallel tests with Ehrlich's reagent after growth in the tryptone medium have confirmed the results. Strains of group VII, type b (*Proteus mirabilis*) are negative to the Ehrlich test after incubation in this medium, but cultures in it give a strong Prussian blue colour with the xanthydrol reagent, which also gives a slightly paler blue colour with cultures in the 'combined' medium. It would appear, therefore, that products of tryptophane metabolism other than indole may be detected with xanthydrol, thus indicating an advantage over the Ehrlich test.

Methods for the detection of urea and choline destruction

Xanthydrol was used by Fosse (1928) as a test for urea and a modification of this method has been used for cultures grown in the 'combined' medium at 37°. Disappearance of urea indicated its hydrolysis by *Klebsiella* (group VI), *Proteus* (group VII), *Past. pseudotuberculosis* (group IX), *Actinobacillus* (group X) and some strains of group II (*Citrobacter*-like) and *Cloaca* (*Aerobacter*) (group V). The results were in agreement with tests on Christensen's urea medium except for certain strains of *Cloaca* (*Aerobacter*) and the 'unclassified type a' strains. It is known that some strains of the *Cloaca* (*Aerobacter*) group are limited to lower temperatures for urease production (Buttiaux, Samaille & Pierens, 1956) and that 'paracolon *Aerobacter*' and 'paracolon intermediate' strains appear to require glucose in the medium (Christensen, 1946). One or both of these factors may have been instrumental in causing the discrepancies observed in the results obtained in the two media for group III and certain group V strains. However, the 'combined' medium has proved to be useful for the detection of urea destruction by strains of the other groups mentioned. Wood & Keeping (1944) have described a test for trimethylamine production from choline for the distinction of strains they described as *Shigella alkalescens* from other members of this genus. *Proteus* and Providence strains are also positive in this test (Buttiaux, Moriamez & Papavassiliou, 1956). During this investigation a spot test for choline has been devised whereby disappearance of choline from the 'combined' medium may be demonstrated after growth of *Proteus* (group VII), Providence (group VIII), certain atypical strains of *Klebsiella* (group IV, type b) and slow or lactose nonfermenting *Escherichia* (group I, types b and c). For comparison choline was determined by the method of Entenman, Taurog & Chaikoff (as modified by Koops, 1958) in the uninoculated medium and after growth of representative strains. The results were analogous to those obtained with the spot test. Ability to destroy choline is of interest in studies of the metabolism of the flora of the alimentary tract in view of the requirement of choline by the host organism. It may also be shown to have value in the differentiation of various groups of the Enterobacteriaceae.

Taxonomy of the groups recognized and their occurrence

Although many of the groups of Enterobacteriaceae that have been recognized here correspond to those described in Report (1958), certain aberrant forms have been observed. Similarly, atypical reactions have been obtained in the Brucellaceae for one type of *Past. pseudotuberculosis* and for the genus *Actinobacillus*.

Enterobacteriaceae. Even though 14 years have elapsed between the work of Borman *et al.* (1944) and Report (1958) on the taxonomy and nomenclature of the Enterobacteriaceae, it appears that, as Borman and his colleagues have said, "The accepted definition . . . fails to point out the manner in which the almost innumerable forms comprising the family intergrade with each other to form an almost continuous series. The degree of interrelationship suggests that we are dealing with organisms having a common phylogeny but existing in a state of evolutionary flux." They also pointed out that "A definition of the family should prepare the student to deal with its subdivisions as arbitrary concepts, utilitarian in purpose, not as sharply-defined, entirely homogeneous categories."

Difficulties have been encountered in the classification of the group *Escherichia* in the course of the present investigations. Some lactose nonfermenting, aerogenic organisms have been recognized (which otherwise comply with the group), in addition to a form anaerogenic on primary isolation (group I, type e) which produced gas after artificial culture and which only differed from strains described by Stuart *et al.* (1943) in its inability to produce indole. These authors considered their lactose non-fermenting strains as variants of 'coliforms', even the anaerogenic types which resembled *Shigella* strains on first isolation.

Variations from published descriptions have also been observed within the other groups of Enterobacteriaceae recognized and some unclassified 'intermediate' groups have been observed.

The metabolism of arginine merits discussion in the case of 'unclassified type a' (group III), *Klebsiella* (group IV) and *Cloaca* (*Aerobacter*) (group V) strains. Strains of these groups gave a negative '2:4-D' reaction after growth in AM. Under aerobic and anaerobic conditions the *Cloaca* (*Aerobacter*) strains described produced NH_3 but not ornithine, and an 'inverse' Barritt test for diacetyl showed that no guanidine compounds remained in the medium after growth of the *Klebsiella* strains. This property of *Klebsiella* strains has not previously been described and may prove to be important for the distinction of the group; but further work is necessary to identify the enzyme system concerned.

Since strains of all the groups described are negative in the test for cytochrome oxidase, there is no fear of confusion with *Aeromonas* spp. (Ewing & Johnson, 1960).

Up to the present, *Escherichia* strains have been most frequent among Enterobacteriaceae isolated from the alimentary tract and faeces of pigs (Bartley & Slanetz, 1960; Fuller *et al.* 1960; Kjellander, 1960), although Phillips (1955) has identified strains of *Proteus vulgaris* and *Pr. mirabilis* from pigs. The present work has shown that organisms typical of the *Escherichia* group are always present and they were dominant on deoxycholate agar plates in all cases except one, in which Providence strains and *Proteus rettgeri* dominated. They may be accompanied by other groups which may appear and disappear from the dominant flora for no apparent reason. These comprise *Escherichia* strains which ferment lactose slowly or not at all, and of which some are able to destroy choline, *Klebsiella*, *Cloaca* (*Aerobacter*), *Proteus* and Providence strains and a few unclassifiable strains intermediate between the *Cloaca* (*Aerobacter*) and *Escherichia* groups.

Brucellaceae. Strains of *Past. pseudotuberculosis* (group IX, types a and b) have been isolated on 7 occasions during this investigation from pigs receiving ration A with or without an antibiotic supplement. Even though the strains tested were not pathogenic for guinea pigs, the morphological, cultural and biochemical characteristics, phage sensitivity and serology of type a strains have allowed them to be identified as typical of the species. Since type b strains showed the characteristic morphology of the species and were motile only at 22°, they have been included as an atypical form although they were not sensitive to the specific phage, were autoagglutinable and differed from type a in biochemical behaviour. Rhamnose fermentation, a character important in the distinction of *Past. pseudotuberculosis* from *Past. pestis* (Devignat & Boivin, 1954), was not exhibited by type b strains. Type b strains could very easily be confused with *Hafnia* (Møller, 1954; Report, 1958) but for their inability to produce H_2S, their production of urease, and their motility only at 22°. No description of strains bearing all the characters of type b has been given previously. A case has been described (Jacotot, Vallée & Le Priol, 1950) of the death of a pig from a *Past. pseudotuberculosis* infection. The organism was isolated from the carcase of an animal from a group of pigs in which the young showed poor growth. However, there does not appear to be any report in the literature of the isolation of *Past. pseudotuberculosis* from pigs in apparently good health.

Organisms morphologically and culturally indistinguishable from *Actinobacillus* (group X) were isolated from pigs on 6 occasions during the present work. Even though a comparison between the results obtained during this work and those described in the literature has been made, difficulties have been encountered in attempting to assign them to a species. They differ from previous descriptions in that they produced gas during carbohydrate fermentation. Further, known species are very ill defined, both on the basis of biochemical behaviour and pathogenicity for laboratory and domestic animals. The results given by Clarenburg (1924) for the pathogenicity test on a guinea pig inoculated with an organism isolated from a piglet which had died from an acute febrile disease, resemble those obtained during the present work. Clarenburg related this organism to those causing joint-ill in foals and described by Magnusson (1929), which have become known as *A. equuli* (Haupt, 1934). However, although resembling Clarenburg's isolates in morphology, the *Actinobacillus* strains described here did not have the viscous nature in culture typical of *A. equuli*. It is interesting to note a correlation between catalase production and lactose fermentation which has permitted a distinction to be made between two types (types a and b). These two types also differed in their ability to hydrolyze urea. It would appear that glucose has an influence on this character since all strains were urease positive in Christensen's medium whereas only type b strains gave a positive reaction in the medium of Hormaeche & Munilla.

It would appear that the two types of *Actinobacillus* strains reported here have not been described previously.

5. SUMMARY

A quantitative and qualitative study has been made of organisms capable of developing on deoxycholate agar from samples of the faeces of pigs receiving standard rations during growth from 20–100 kg. Samples of the contents of the alimentary tract were similarly examined at slaughter. Average counts of $4{\cdot}3\times10^4$, $1{\cdot}5\times10^6$ and $6{\cdot}5\times10^6$/g of gastric, intestinal and caecal contents, respectively, and $1{\cdot}7\times10^7$/g of faeces were obtained.

A total of 1,670 strains of facultative anaerobes was studied. A primary classification of isolates has been made on the results of glucose fermentation in a glucose agar medium, indole and lysine decarboxylase production and growth at 45° in the presence of bile salts. Further differentiation into 10 groups has been carried out on the basis of tests recommended in Report (1958). Modifications have been described for phenylalanine deaminase, arginine dihydrolase and acetoin production after growth in mineral salts-yeast extract media, for urea and choline destruction and for indole production.

Aerogenic *Escherichia* strains showing prompt and slow fermentation of lactose and anaerogenic strains showing prompt fermentation have been recognized. Lactose

nonfermenting strains which otherwise resemble *Escherichia* and which are aerogenic or become so on laboratory cultivation have also been observed. *Citrobacter*-like, *Klebsiella*, *Cloaca* (*Aerobacter*), *Proteus*, Providence and some unclassifiable strains have also been isolated from high dilution plates. *Escherichia* strains were dominant among Enterobacteriaceae groups except in one case, when they were outnumbered by Providence strains accompanied by *Proteus rettgeri*.

Pasteurella pseudotuberculosis strains, nonpathogenic for guinea pigs but otherwise typical, have been isolated in several instances. An atypical form of this species differing in its ability to produce gas in a nutrient agar medium at 30° and 22° and acetoin at 22°, and in its fermentation of carbohydrate, has also been isolated. Catalase positive, lactose negative and catalase negative, lactose positive strains of the genus *Actinobacillus*, each showing distinctive biochemical characteristics but differing from known species of the genus by their aerogenic character in media containing a fermentable carbohydrate, are described. Both *Past. pseudotuberculosis* and *Actinobacillus* strains have been isolated from apparently healthy animals which showed normal growth.

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EXPLANATION OF PLATE

Plate 1 (a). Electron micrograph of an organism of group IX (*Pasteurella pseudotuberculosis*) from the faeces of a pig, × 15,000.

Plate 1 (b)–(d). Micrographs of *Actinobacillus* spp. from LAPTg5 medium, × 1,400.
(b). Group X type b from the faeces of a pig.
(c). Strain of *Actinobacillus lignieresii* received from Dr. Phillips.
(d). Strain of *Actinobacillus equuli* received from Dr. Vallée.

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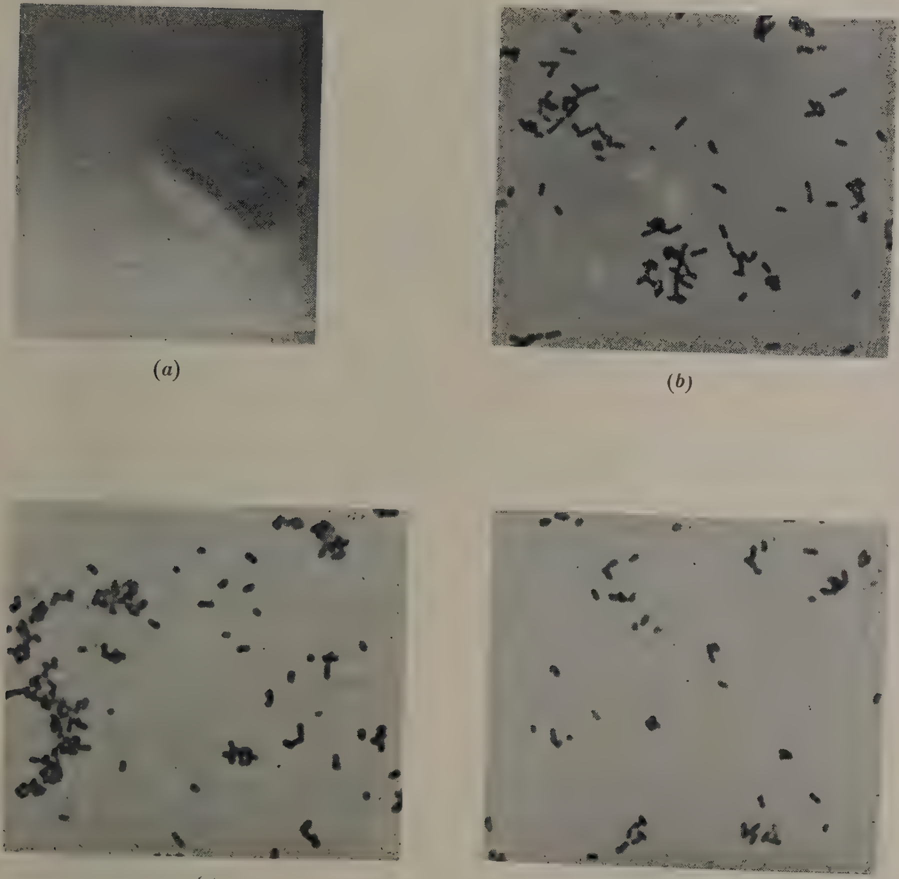

(*a*) (*b*)

(*c*) (*d*)

Anne B. Dickinson & G. Mocquot. — Studies on the Bacterial Flora of the Alimentary Tract of Pigs. I. Enterobacteriaceae and other Gram-negative Bacteria. Plate 1

Raibaud, P., Caulet, M., Galpin, J. V. & Mocquot, G. (1961). *J. appl. Bact.* **24** (3), 285–306

(Symposium on Bacteria of the Intestine: Paper IV)

STUDIES ON THE BACTERIAL FLORA OF THE ALIMENTARY TRACT OF PIGS. II. STREPTOCOCCI: SELECTIVE ENUMERATION AND DIFFERENTIATION OF THE DOMINANT GROUP

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1. Introduction

Opinions have differed among previous workers who have studied members of the genus *Streptococcus* isolated from the alimentary canal or faeces of pigs (Table 1). Some workers have found that *Streptococcus faecium* or *Strep. faecium* and *Strep. faecalis* and its varieties are the most frequent, whereas others have found *Strep. bovis* or *Strep. equinus* to be the most numerous. In addition, many closely related strains have been recognized but have not been fully classified.

Table 1 also indicates how the methods used in these studies have varied. Morelis (1958), Kjellander (1960) and Mieth (1960) have referred to 29 different selective media for the enumeration of streptococci belonging to Lancefield group D. This long list of media gives an impression of the complexity of the problem of the selective enumeration of this group when it forms part of a heterogeneous microbial population.

A recent publication (Kenner, Clark & Kabler, 1961) has stressed the particular difficulty of the quantitative isolation of certain streptococci which, according to Kjellander (1960), possess "... the poorest growth tendencies at 45°, 10° or in 6·5% NaCl, as well as the lowest biochemical activity."

Table 1. *Résumé of previous studies on streptococci isolated from the alimentary tract of pigs*

Authors	Source of sample	No. of samples	Media used	No. of strains	Identity	Distribution of	
						Strains	Samples
Barnes & Ingram (1955)	Colon	21	TlTG agar	90	*Strep. faecium*	40	
					Unclassified	50	
Fewins et al. (1957)	Caecum Faeces		RCM and horse blood agar	57	Unclassified	56	
					Strep. bovis	1	
Raibaud & Caulet (1957)	Caecum Faeces		AVPG agar	54	*Strep. bovis* group	53	
					Strep. faecium	1	
Buttiaux (1958)	Faeces	52	TlTG agar		*Strep. faecalis* and varieties		10
					Strep. faecium		48
					Strep. bovis		3
Moutoussis et al. (1958)	Faeces	10	TlTG agar		*Strep. faecalis*		1
					Strep. faecium		5
					Strep. bovis		1
Morélis (1958)	Faeces	15	Aesculin-azide agar	15	*Strep. faecalis* and biotypes		15
Fuller et al. (1960)	Faeces		RCM and horse blood agar	252	*Strep. faecalis*	7	
					Strep. durans	8	
					Strep. faecium	5	
					Strep. equinus	160	
					Strep. bovis	2	
					Unclassified	56	
Mieth (1960)	Faeces	100	TKT agar	327	*Strep. faecalis* and varieties	36	22
					Strep. faecium	100	56
					Strep. bovis and variants	173	88
					Atypical enterococci	18	
Kjellander (1960)	Faeces	47	Azide-sorbitol agar	47	*Strep. bovis*		15
Bartley & Slanetz (1960)	Faeces		Medium of Slanetz	136	*Strep. faecium*, *Strep. equinus* and *Strep. faecalis* biotypes		
Kenner et al. (1960)	Faeces	5	KF medium	100	Enterococcus group	10	
					Strep. bovis group	32	
					Strep. equinus group	24	
					Enterococcus biotypes	34	

The object of this work was to study a certain number of biochemical, physiological and serological characters of the dominant streptococci present in the caecum and faeces of pigs and to devise a selective medium (the thirtieth!) for the efficient enumeration of these streptococci in the presence of the numerous other microbial genera which constitute the 'normal' flora of the pig.

2. MATERIALS AND METHODS

All concentrations are given as % (w/v) unless otherwise stated.

(a) *Samples*

Samples were taken over a period of 3 years from the gastric and caecal contents and faeces of Large White and Piétrain pigs between 2 and 7 months of age, housed in individual pens in two piggeries at the C.N.R.Z. In addition, some samples were taken from calf rumen contents, rat, chicken and horse caecum contents and from fresh human and duck faeces. The rations (A, with and without antibiotic, B and C) received by the pigs, the method of slaughter, and the preparation for bacteriological examination have been described by Dickinson & Mocquot (1961).

(b) *The cultivation and selective enumeration of streptococci*

Media. Unless mention is made to the contrary, the basis of media was LAPT, which for the growth and selective enumeration of streptococci contained: Bacto autolyzed yeast (Difco), 1·0; peptone (Evans), 1·5; Bacto tryptone (Difco), 1·0; in de-ionized water. The pH was adjusted to 7·6–7·8 before a primary sterilization at 120° for 20 min. The medium was then filtered under reduced pressure after addition of a filtering aid (Hyflo Super-Cel, Johns-Manville), tubed and sterilized at 118° for 20 min. The following media were prepared with the LAPT base: LAPTgl and LAPTg5, containing 0·1% and 0·5% of glucose, respectively; GAPTgl and GAPTsl, 2% agar media containing 0·1% of glucose and sucrose, respectively; GeAPTgl and GeAPTg50, 20% gelatin media containing 0·1% and 5% of glucose, respectively; LAPT0·4, 0·6 and 0·8, consisting respectively of 4, 6 and 8 volumes of LAPT diluted to 10 volumes with de-ionized water.

Selective enumeration. The media and techniques described by Barnes (1956*b*) (TlTG medium), Slanetz & Bartley (1957) (M-Enterococcus agar, MEA), and Kenner *et al.* (1961) (KF medium) have been used. Surface inoculations of 0·1 ml of each decimal dilution were made on these media. In some cases, instead of using this technique, 1 ml of each dilution was poured with the three different media. Incubation was for 48 hr at 37°.

A selective medium (AGAT) was devised. This medium is composed of GAPTsl as the nutrient base to which sodium azide, sodium glutamate, acridine orange and triphenyl tetrazolium chloride (TTC) are added in two aqueous solutions. Solution 1 contains 0·45% of sodium azide (Merck) and 15% of sodium glutamate and is sterilized by boiling for 30 min in a waterbath. Solution 2 contains 0·015% of acridine orange (Gurr) and 0·075% of TTC (Merck) and is sterilized by Seitz-filtration and kept in the dark. Inoculation of the AGAT medium was carried out in the following manner. A volume of 1 ml of the required decimal dilution of the sample was added to a tube (18×180 mm) containing 1 ml of solution 1 and 0·2 ml of solution 2 and the tube was placed for 5 min in a waterbath thermostatically controlled at 37°. To this tube 15 ml of melted GAPTsl at 48° were added and the contents were immediately transferred to a Petri dish. Incubation was for 48 hr at 37°. It is important to stress the necessity of following the technique described, which allows preliminary contact between the bacteria and the inhibitors at concentrations about 8 times those in the final AGAT medium.

(c) *Bacteriological methods for differentiating the dominant streptococci*

Origin of strains. Colonies were isolated from high dilution AGAT plates which had been poured with decimal dilutions of 7 samples of caecal contents and 3 samples of faeces taken from 6 Large White and 2 Piétrain pigs weighing 90–100 kg. Only plates containing 5–50 well separated colonies were selected for examination. A total of 100 strains of nonmotile cocci occurring in pairs and chains of variable length and form, recognized by microscopical examination of a drop of unstained culture, were retained for identification. They were checked for purity and examined for haemolysis on nutrient agar (Difco) containing 5% (v/v) of defibrinated horse blood before identification. A 5–9 hr LAPTg5 culture (inoculated from an overnight culture) incubated at 37° was used to inoculate the various media described below. A 2% inoculum was used, except for the precipitin, heat resistance and acetoin from citrate tests for which 4% was used. The media employed for testing catalase activity and action on TTC were streaked over the surface with one drop of culture. The latter test was carried out after incubation for 18 hr at 37°; all other media were incubated for 8 days at 37°.

The strains were identified on the results of the following tests:

Serological methods

Precipitin tests were carried out as follows. Hydrochloric acid extracts prepared from 18 hr cultures in Lab-Lemco broth containing 1% of glucose were concentrated with ethanol by the technique described by Shattock (1949). These extracts were tested in capillary tubes against group D sera prepared by the Institut Pasteur, by Burroughs Wellcome and, in some cases, by Difco. The tubes were observed for 20 min.

Physiological methods

For these tests either a standard medium or a medium which will ensure the optimal growth of the organism to be studied may be chosen. The latter course was adopted for the present work. Since the maximum turbidity of a culture of one strain, expressed as the optical density at 545 mμ (Photomètre Eral) after incubation for 24 hr at 37°, was 0·86 in LAPTg5 compared with 0·51 in glucose-Lemco broth, LAPTg5 was chosen for studies on the behaviour of strains in the following three tests.

Growth at 10°, 45° *and* 48°. Cultures incubated in thermostatically controlled water-baths accurate to ±0·1° were examined for turbidity and pH changes.

Resistance to inhibitory substances. Media containing the following substances were dispensed in screwcapped tubes: 2%, 4% and 6·5% of sodium chloride (Sherman & Stark, 1934); 2% of sodium taurocholate (Prolabo) (Wheater, 1955); 0·1% of Teepol (Shell-Berre) (Jameson & Emberley, 1956); 0·04% of potassium tellurite (Skadhauge, 1950), prepared by adding 0·2 ml of Seitz filtered 1% aqueous K_2TeO_3 (B.D.H.) to 5 ml of the sterile medium. Growth was judged by turbidity and pH change.

Growth at pH 9·6. The method of Shattock & Hirsch (1947) was used, except that LAPTg5 was employed. It was sterilized by Seitz filtration and immediately inoculated. Growth was judged by turbidity.

Utilization of sodium lactate. This was studied in LAPT containing 1% (v/v) of lactic acid (practical), adjusted to pH 6·8 (Foster, Garey & Frazier, 1942). Growth was judged by turbidity.

Heat resistance. This was studied at 63° for 30 min by the method of Abd-el-Malek & Gibson (1948). Strains giving less than 20 colonies after inoculation into GAPTsl following heat treatment were considered not to be heat resistant.

Reaction in litmus milk. The sequence of changes (acid production, coagulation and reduction of the litmus) in this medium was noted.

Biochemical methods

Catalase production. This was determined on GAPTgl slope cultures with H_2O_2 (10 vol).

Nitrate reduction. This was determined in LAPTgl containing 0·1% of sodium nitrate (Rogosa, 1961). Nitrite was tested for by the Griess-Ilosvay method, and nitrate was tested for at the end of the incubation period by testing for nitrite after adding zinc powder.

Action on TTC. This was observed by the method described by Barnes (1956*a*) using TTC obtained from Merck.

H_2S production. This was determined with lead acetate paper on cultures in LAPTgl containing either 0·01% of L-cysteine or 0·05% of sodium hyposulphite (Barnes & Shrimpton, 1957).

CO_2 production from glucose. This was determined by a modification of the test described by Gibson & Abd-el-Malek (1945), using inoculation into GeAPTg50 in Veillon tubes.

Production of dextran or related substances. The medium described by Bailey & Oxford (1958) was used. This contained 0·004 M potassium carbonate and 0·184 M sodium acetate and was sterilized by Seitz filtration. A positive reaction was indicated by a sustained turbidity in the medium after incubation and the formation of an abundant precipitate in the supernatant of the centrifuged culture after ethanol treatment.

Formation of acetoin from citrate. The medium described by Swartling (1951) was used and after incubation acetoin was tested for by Barritt's (1936) modification of the Voges-Proskauer test.

Decarboxylation of L-tyrosine. This was examined (Sharpe, 1948) after growth in LAPTg5 with 0·5% of L-tyrosine added. The test was considered to be negative when the difference in pH between LAPTg5 with and without added tyrosine was less than 0·8.

Hydrolysis of urea. The following three methods were used to determine whether urea had been hydrolyzed in LAPTg5 containing 0·6% of urea (added to the sterile medium after Seitz filtration): the final pH of media with and without urea; NH_3 production by the spot test with Nessler's reagent, described by Niven, Smiley & Sherman (1942); the presence of residual urea by the xanthydrol test (Fosse, 1928).

The last mentioned test was done by adding 1 ml of 1% xanthydrol in acetic acid to 0·5 ml of culture and then placing the tube for 1 min in boiling water. When urea is present a heavy precipitate of dixanthyl urea is formed, and no precipitate indicates that the urea has been hydrolyzed.

Production of NH_3 from arginine. The medium and method of Niven, Smiley & Sherman (1942) were used.

Hydrolysis of gelatin. This was indicated by liquefaction after growth in GeAPTgl.

Hydrolysis of aesculin. This was indicated by a blackening after the addition of $FeCl_3$ solution (Cunningham, 1947, p. 36) to a culture in LAPTgl plus 0·1% of aesculin (B.D.H.).

Hydrolysis of soluble starch. This was determined after growth in a medium similar to that described by Seeley & Dain (1960) but without azide or agar, by comparing the final pH of the medium with and without starch, and by testing for starch with a modification of the iodine test. For this modification 0·2 ml of $FeCl_3$ solution (Cunningham, 1947, p. 36) and 0·2 ml of Lugol's iodine solution were added both to 5 ml of culture and to 5 ml of the uninoculated medium. After standing for 3 hr at room temperature, the colours in the two tubes were compared. The violet colour obtained in the control tube persisted for 24 hr, whereas cultures in which starch had been hydrolyzed decolourized or became reddish violet.

Hydrolysis of sodium hippurate. This test (Ayers & Rupp, 1922) was determined after growth in a medium similar to that described by Seeley & Dain (1960) but without starch and azide and containing 2% of sodium hippurate (B.D.H.). The presence of benzoic acid was revealed in the medium by adding 2 drops of 50% (v/v) H_2SO_4.

Carbohydrate fermentation. This reaction determined in LAPT0·4 containing 0·002% of chlorophenol red (Wheater, 1955) and 0·5% of the following 'sugars': D-ribose, L- and D-arabinose, D-xylose, D-mannose, D-galactose, D-fructose, L-sorbose, D-maltose D-glucosamine hydrochloride and D-lactose (all added after Seitz filtration), L-rhamnose (hydrate), D-trehalose, D-sucrose, D-cellobiose, D-melibiose, D-melezitose (hydrate), raffinose (practical), soluble starch, inulin, *i*-inositol, salicin, amygdalin, glycerol, *i*-adonitol, D-mannitol, D-sorbitol and *i*-dulcitol. Tests for carbohydrate fermentation were considered to be positive when the final pH was less than 5·6. This corresponded with an indicator change from red to orange yellow. A control tube of LAPT0·4 was inoculated with each strain for comparison.

(d) *Bacteriological methods for studies on urea hydrolysis*

Origin of strains. A collection of 424 strains, isolated by means of the AGAT medium, was used for this investigation. Of the dominant streptococci 415 strains, including the 100 strains selected for detailed information, were isolated from the stomach (1 strain), caecum (206 strains) and faeces (208 strains) of pigs. For comparison 9 strains which had been isolated from samples of caecal contents or faeces taken from the other animals cited and from human faeces, and identified following the methods described, were included.

Media. Inoculations were made into the following media containing 0·6% of urea: LAPTg5, with and without 2% of sodium chloride; LMPTg5, a medium containing 0·5% of meat extract (Lab-Lemco) instead of yeast autolysate; LAPT0·4, 0·6 and 0·8, with 0·1% of glucose; and LAPT with 0·1, 0·2, 0·3, 0·4, 0·5, 1·0, 1·5, 2·0, 2·5 and 3·0% of glucose. Media made by separately adding 0·1 M thiourea, L-citrulline, orotic acid (Fluka) neutralized with NaOH, L-arginine monohydrochloride, L-glutamine and D-glucosamine hydrochloride to LAPTg5 were inoculated for comparison. All the strains were inoculated into LAPTg5 with and without urea, whereas the other media were inoculated with various strains selected from the 100 chosen for identification. Incubation was for 8 days at 37° unless mention is made to the contrary.

Methods. Of the 3 methods described for the detection of urea hydrolysis, the first two were also used for detecting the breakdown of citrulline, orotic acid, glutamine and glucosamine. For the detection of the breakdown of thiourea, however, only the pH method could be used because Nessler's reagent precipitates with this compound.

3. Results

(a) *Differential characters of the dominant streptococci*

The serological, physiological and biochemical characters common to the 100 strains of streptococci studied are given in Tables 2 and 3, and some of their variable physiological and biochemical characters are shown in Table 4.

Table 2. *Some serological and physiological characters common to* 100 *strains of dominant streptococci from the alimentary tract of pigs*

Test		Result
Precipitin test with group D antiserum		+
Growth* at	10°	−
	45°	+ or I
	48°	−
Growth* in presence of	6·5% NaCl	−
	2% NaCl	+ or I
	2% sodium taurocholate	+
Growth at pH 9·6		−
Growth in lactate medium		−
Inhibition by 0·04% potassium tellurite		+
Survival at 63° for 30 min		−

* Growth detected by pH change: +, pH 5·2; I, pH < 6·0.

The precipitin test with group D sera obtained from the Institut Pasteur and from Burroughs Wellcome were distinctly positive after 3 to 5 min with antigens prepared by the ethanol method described by Shattock (1949). However, the same extracts gave a negative result with the group D serum obtained from Difco.

There was a good correlation between the iodine test devised for starch hydrolysis and the final pH in the medium. The final pH in the control medium was 6·85±0·15, and the iodine test was negative. Cultures which decolourized in less than 1 hr after addition of the reagent showed a final pH of 5·5–6·2, and a reddish violet colour corresponded to a pH of 6·2–6·4. It may be seen that starch was fermented by only 3% of the strains (Table 4), whereas it was hydrolyzed by all of them (Table 3). The final pH of cultures of all strains in LAPT0·4 without added carbohydrate was 7·2–7·4. Therefore neither the inoculum nor the basal medium provided enough carbohydrate to give rise to positive reactions in the tests for carbohydrate fermentation.

Table 3. *Some biochemical characters common to* 100 *strains of dominant streptococci from the alimentary tract of pigs*

Test		Result	Test		Result
Hydrolysis of	urea	+	Fermentation of	Glycerol	—
	L-arginine	—		Xylose	—
	starch	+		L-Arabinose	—
	gelatin	—		D-Arabinose	—
Haemolysis in horse blood agar		+		Ribose	—
H_2S formation from cysteine and hyposulphite		—		Adonitol	—
Catalase production		—		Rhamnose	—
Nitrate reduction		—		Glucose	+
Aerobic TTC reduction at pH 6·0		—		Mannose	+
Litmus milk reduction before acidification		—		Sorbose	—
Formation of L-tyrosine decarboxylase		—		Dulcitol	—
Production of CO_2 from glucose		—		Inositol	—
Production of dextran from sucrose		—		Sucrose	+
				Maltose	+
				Cellobiose	+
				Melezitose	—
				Inulin	—
				Salicin	+
				Glucosamine	—

Table 4. *Some variable characters among* 100 *strains of dominant streptococci from the alimentary tract of pigs*

Test	Result	Proportion (%) giving result indicated
Growth in presence of 4% NaCl	—	94
Growth in 0·1% Teepol	+	98
Sodium hippurate hydrolysis	—	69
Acetoin formation from citrate*	+ or I	84
Starch fermentation	—	97

* Reaction in Barritt test: +, strong; I, weak.

Independent of the diet or the breed of pig from which the strains originated, a variable percentage attacked other carbohydrates (Table 5). It may be noted in particular that variable results for mannitol, lactose and melibiose fermentations were obtained with strains from each of the 10 samples studied.

Table 6 shows that 29 strains of streptococci isolated from the same dilution of caecal contents could be divided into 17 types which differ one from another with regard to their behaviour towards at least one carbohydrate. However, one type was more frequent than the rest. Strains of this type fermented raffinose, melibiose, fructose, galactose, aesculin and amygdalin, as well as the carbohydrates fermented by all strains. It was also the most frequent type among the 100 strains studied and was isolated from 9 out of the 10 samples examined.

The 100 strains may be divided into 38 types, each showing a different fermentation pattern. Reactions towards only 4 of the carbohydrates appeared to be linked in pairs, i.e. all strains fermenting sorbitol also fermented mannitol and all those fermenting melibiose also fermented raffinose; but in both cases the converse was not true. There are correlated reactions in 99% of the cases for aesculin and amygdalin hydrolysis, only 2% of the strains fermented galactose without fermenting fructose, and galactose and lactose fermentation do not appear to be linked. If the 4 pairs of characters—fermentation or not of mannitol, galactose, raffinose and lactose—are considered, it may be seen that 14 out of the 16 combinations possible when the characters are considered independently one from another were observed. The two combinations which were not observed are: lactose negative, mannitol and raffinose

Table 5. *Variations in carbohydrate fermentation by* 100 *strains of dominant streptococci from the alimentary tract of pigs*

Carbohydrate	Fermentation results* obtained with indicated nos. of strains from									
	Faeces[1] 8†	Faeces[1] 4†	Faeces[1] 4‡	Caecum[2] 8§	Caecum[3] 10§	Caecum[2] 29‡	Caecum[4] 21‡	Caecum[3] 5§	Caecum[3] 6†	Caecum[4] 5§
Sorbitol	−(7)	−(2)	−(2)	−(7)	−	−(26)	−(17)	−	−(5)	−
Mannitol	−(5)	+(3)	−(2)	−(6)	−(8)	−(20)	−(15)	−(4)	+(4)	+(4)
Aesculin	+	+(3)	+	+(7)	+(9)	+(26)	+(18)	+(4)	+	−(3)
Amygdalin	+	+(3)	+	+(6)	+(9)	+(26)	+(18)	+(4)	+	−(3)
Galactose	+(4)	+(3)	+	+	+(6)	+(18)	+(20)	+	+	+
Fructose	+	+(3)	+	+	+	+	+	+	+	+
Lactose	−(7)	−(3)	−(3)	−(6)	−(8)	−(23)	−(17)	−(3)	−(4)	−(3)
Melibiose	+(4)	+(2)	+(2)	−(5)	+(7)	−(15)	−(11)	+	+(5)	−(4)
Trehalose	−(7)	−	−	−(7)	−(9)	−	−	−	−	−
Raffinose	+(7)	+	+	+(6)	+(9)	+(23)	(+18)	+	+(5)	+(3)

* The nos. of strains which gave the indicated reaction are shown in brackets. † Isolated from 10^{-8} dilution. ‡ Isolated from 10^{-7} dilution. § Isolated from 10^{-5} dilution. [1] From a Large White pig fed on diet A (see text) plus aureomycin. [2] From a Piétrain pig fed on diet B (see text). [3] From a Large White pig fed on diet A (see text). [4] From a Large White pig fed on diet C (see text).

Table 6. *The incidence of strains of dominant streptococci with different fermentation patterns among* 29 *isolates from the same dilution of a single sample* (*a*) *and* 100 *isolates from* 10 *samples* (*b*) *from the alimentary tract of pigs*

	Fermentation patterns*																	Positive strains (%)	
																		(a)	(b)
Sorbitol	+	+	+															10·5	14
Mannitol	+	+	+	+	+	+	+	+										31	36
Aesculin	+	+	+	+	+	+	+	+	+	+	+	+	+	+				89·5	91
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+				89·5	90
Galactose			+			+	+	+				+	+	+		+	+	62	79
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100	98
Lactose	+							+		+			+					17	23
Melibiose					+						+			+	+			48·5	52
Trehalose																		0	3
Raffinose		+			+		+	+		+	+	+	+	+	+		+	83	85
Strains (%) of each type from (a)	3·5	3·5	3·5	3·5	7·0	3·5	3·5	3·5	3·5	3·5	10·5	3·5	10·5	27	3·5	3·5	3·5		
(b)	1	1	1	2	3	2	2	1	1	1	5	7	6	26	1	2	2		

* For clarity of presentation negative signs have been omitted.

positive, galactose positive; and lactose negative, mannitol and raffinose positive, galactose negative. Finally, trehalose fermentation, a character which has rarely been encountered, does not appear to be linked to action on any other carbohydrate.

Table 7. *Carbohydrate fermentation by 30 substrains from one of 100 strains of dominant streptococci from the alimentary tract of pigs*

Test		Results* and nos. of strains			
Fermentation of	Galactose	+	+	—	+
	Raffinose	+	+	—	—
	Amygdalin	+	—	—	—
		1	1	1	27

* All the substrains behaved identically towards other carbohydrates.

Table 8. *A comparison of some physiological and biochemical characters of dominant streptococci from the alimentary tract of pigs with those of streptococci described by other workers*

Test		Results* and strains (%) giving indicated reaction according to			
		Present authors	Fuller *et al.* (1960)	Mieth (1960)	Breed *et al.*† (1957)
Precipitin test with group D antiserum		+	+	+	+
Growth in presence of 6·5% NaCl		—	— (99)	— (95)	—
Inhibition by 0·04% potassium tellurite		+	+	+	
Haemolysis (horse blood)		—	—	—	—
Hydrolysis of	Urea	+			
	L-Arginine	—	—	— (96·5)	—
	Gelatin	—	—	—	—
	Sodium hippurate	— (69)		— (99)	±
	Starch	+	— (99)		+
Starch fermentation		— (97)		+ (93)	
Action on litmus milk		AC (12) ACR (12)	A or AC (21) ACR (3) ARC (2·5)	A, AR or ACR (41) RAC or RA (8)	usually ACR
Fermentation of	Glycerol	—	— (99)	— (97)	—
	L-Arabinose	—	— (99)	— (98·5)	±
	Glucose	+	+	+	+
	Sorbose	—		— (94)	—
	Lactose	— (77)	— (74)	— (51)	+
	Sucrose	+		+ (97)	+
	Trehalose	— (97)		— (78·5)	±
	Sorbitol	— (86)		— (82)	±
	Mannitol	— (64)	+ (71)	— (52·5)	±
	Raffinose	+ (85)	+ (82)	+ (76·5)	+
	Inulin	—		— (97)	±
	Aesculin	+ (91)		+ (99)	+
Nos. of strains		100	216	187	

* Reactions: +, positive; —, negative; ±, positive or negative; A, acid; C, clot; R, reduction.

† *Streptococcus bovis.*

Because the occurrence of such a diversity of fermentation patterns might be due either to the appearance of an adaptive enzyme system in the culture or to the presence of a mixed bacterial population originating from a single colony, the two following experiments were carried out. First, strain 259/13 was successively subcultured 27 times with a 0·2% inoculum in LAPTg5 at 12–14 hr intervals. The 28th subculture was inoculated into the various carbohydrate media employed. All results from fermentation tests were identical to those obtained during the initial characterization. Second, decimal dilutions of a 6 hr culture in LAPTg5 of strain 248/1 were inoculated into GAPTsl. Then 30 colonies from the 10^{-6} dilution were subcultured, and the 30 substrains thus obtained inoculated into the carbohydrate media. Table 7 shows that these strains could be divided into 4 types with different fermentation patterns, the distribution being uneven.

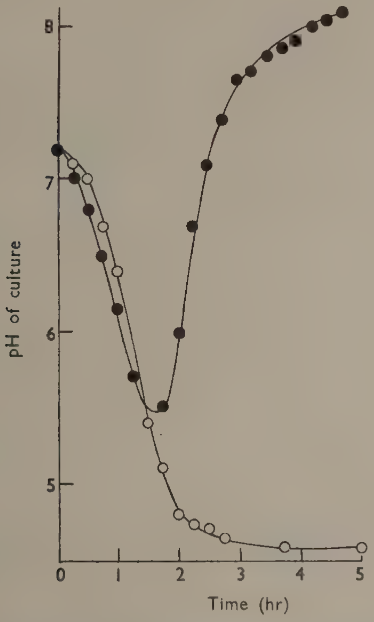

Fig. 1. The changes in pH in LAPT containing 0·5% of glucose, with urea present (full circles) and absent (open circles), during the growth of a urease positive strain from the caecal contents of a pig.

This variation in carbohydrate fermentation would not appear to be peculiar to the strains isolated from pigs during this investigation because the 403 strains studied by Fuller *et al.* (1960) and Mieth (1960) differed in their ability to ferment sorbitol, mannitol, lactose and raffinose (Table 8).

(b) *Urea hydrolysis*

Demonstration of urea hydrolysis. The three urea hydrolysis tests were carried out on samples taken at 15 min intervals over a period of 5 hr and daily for 2 days from cultures of one of the strains of streptococci in LAPTg5 and LAPTg5-urea. After incubation for 2 hr in LAPTg5-urea, both the Nessler test for NH_3 and the xanthydrol test were negative but the pH was between 5·5 and 6·0. This pH was higher than that in LAPTg5 (Fig. 1), therefore it would appear that hydrolysis of urea had taken place. The Nessler test was positive when the pH in LAPTg5-urea was higher than 6·0, whereas the xanthydrol test was positive only after incubation for 48 hr at 37°.

The effect of glucose in the growth medium. The work of Christensen (1946) on urea hydrolysis by certain members of the Enterobacteriaceae has drawn attention to the important effect of the presence of glucose in the medium on urea hydrolysis. Table 9 shows that for one representative strain in LAPT and LAPT-urea the following applied. First, with a concentration of 0·1% of glucose the difference between the final pH of cultures in these media indicated that part of the urea had been hydrolyzed, even though the Nessler and xanthydrol tests were both negative. Second, with a concentration of 0·2% of glucose, all the urea had not been hydrolyzed since the Nessler test was positive and the xanthydrol test was negative. Finally, with increase of the glucose to 0·3% and above both the Nessler and xanthydrol tests were positive, and the difference between the pH in LAPT and LAPT-urea decreased with increase in the glucose concentration.

Table 9. *The effect of glucose on the hydrolysis of urea by one of* 100 *strains of dominant streptococci from the alimentary tract of pigs*

Medium*	Test	Concn of glucose (%)									
		0·1	0·2	0·3	0·4	0·5	1	1·5	2	2·5	3
LAPT	pH	6·95	6·30	5·40	4·85	4·55	4·15	4·15	4·20	4·20	4·20
	NH_3†	−	−	−	−	−	−	−	−	−	−
LAPT + 0·6% urea	pH	7·55	8·70	8·90	9·00	9·00	7·75	7·20	7·20	5·30	4·80
	NH_3†	−	+	+	+	+	+	+	+	+	+
	Xanthydrol test	−	−	+	+	+	+	+	+	+	+

* Initial pH 7·4; inoculum 0·2%; incubation for 8 days at 37°. † Ammonia detected by spot test.

The effect of the composition of the growth medium. A *Streptococcus* strain which hydrolyzed urea in LAPTg5 also exhibited this character in LAPTg5-NaCl and in LMPTg5. This strain only partially hydrolyzed urea in LAPT containing 0·1% of glucose (Table 9). Since Gibbons & Doetsch (1959) have said that "Most bacteriological culture media are rich in organic nitrogenous materials, and subsequently could inhibit urease formation . . . . ", it was of interest to see whether the Nessler and xanthydrol tests would be positive in diluted LAPT containing 0·1% of glucose. The same strain was therefore inoculated into LAPT and LAPT0·4, 0·6 and 0·8, each containing 0·6% of urea and 0·1% of glucose. However, at the end of the incubation period, samples from all the media gave negative reactions with the Nessler and xanthydrol reagents.

The action of urease positive streptococci on certain substances related to urea. A *Streptococcus* strain capable of hydrolyzing urea did not hydrolyze either thiourea or citrulline (monosubstituted urea) or sodium orotate (disubstituted urea) under the same conditions. Arginine, which possesses a guanidine group, glucosamine and glutamine were also not hydrolyzed.

The effect of successive subculture in the presence of urea. A strain of *Streptococcus* (258/10) which was able to hydrolyze urea on first isolation retained this character even after 100 successive subcultures in LAPTg5 at intervals of 12–14 hr.

The incidence of urease positive strains of streptococci in the samples examined. The 415 strains described in section (d) of the Methods hydrolyzed urea. However, with 9 strains the xanthydrol test was negative, the Nessler test was positive, and the final pH approximately 8·0. These 9 strains had not, therefore, hydrolyzed all the urea in the medium. In addition, the xanthydrol and Nessler tests were negative at the end of the incubation period in cultures of one strain (248/1), although the final pH was higher (5·2) in LAPTg5-urea than in LAPTg5 (4·8).

The effect of successive subculture in the presence of urea. Strain 248/1 was successively subcultured in LAPTg5-urea with a 0·2% inoculum at intervals of 12–14 hr. Even though the Nessler and xanthydrol tests were negative at the first subculture, at the second the final pH was 8·35 and a positive reaction was then obtained for the Nessler test although the xanthydrol test was still negative. At the third subculture the final pH was 8·45 and the Nessler and xanthydrol tests were both positive. With the object of determining whether other types or species of streptococci originating from the alimentary tract and faeces of pigs and other animals could acquire the ability to hydrolyze urea, a few strains were successively subcultured in LAPTg5-urea at intervals of 9–15 hr. The following were chosen: 1 strain each of *Strep. faecalis* from human faeces, *Strep. faecalis* var. *liquefaciens* from rat caecal contents and *Strep. durans* from chicken caecal contents; 1 strain from pig faeces, related to *Strep. faecium* and having the characters of the unclassified type 38 described by Sharpe & Fewins (1960); and 5 strains resembling *Strep. bovis*, the characters and origins of which are given in Table 10. After 33 subcultures the final pH in LAPTg5 with and without urea was the same and the Nessler and xanthydrol tests were negative. Therefore none of these strains had acquired the ability to hydrolyze urea.

(c) *The selective enumeration of streptococci from the alimentary tract and faeces of pigs by means of the AGAT medium*

Description of the colonies observed in AGAT medium. The following colony types could be recognized in AGAT medium. The descriptions have been based on the results obtained from the examination of approximately 600 plates.

Type 1 colonies (Plate 1a) were lenticular (diameter 1–1·2 mm) and had rose coloured centres when there were fewer than 100 colonies/plate; when they were more numerous, however, the colonies were white and small. These types are typical of the colonies of streptococci appearing on high dilution plates from samples of the intestinal contents and faeces of pigs. Because members of the *Strep. bovis* group (Table 10) give colonies of this nature, type 1 colonies were designated as typical of *Strep. bovis*. They may easily be distinguished from the other colony types.

Table 10. *Some physiological and biochemical characters* of streptococci from various animals*

Test		Strain no. and source				
		1 Calf rumen	2 Rat caecum	3 Duck faeces	4 Chicken caecum	5 Horse caecum
Precipitin test with group D antiserum		+	+	+	+	−
Growth at 45°		+	+	+	−	+
Growth in presence of 4% NaCl		+	+	−	−	−
Growth in presence of 0·1% Teepol		+	+	+	+	−
Formation of acetoin from citrate		+	−	−	+	+
Formation of dextran from sucrose		+	−	−	−	−
Fermentation of	L-Arabinose	−	−	−	+	−
	Sorbitol	−	−	−	−	+
	Mannitol	−	−	−	−	+
	Lactose	+	−	−	−	+
	Melibiose	+	−	−	−	+
	Trehalose	−	+	+	−	+
	Raffinose	+	+	+	−	+
	Inulin	+	−	−	−	+
	Glucosamine	+	−	+	+	+

* All strains fermented galactose, fructose and amygdalin and were identical in all other characters to those isolated from pigs.

Type 2 colonies were lenticular (diameter 1–1·5 mm), with red centres and with or without a surrounding white ring. Single or double lens shaped colonies with no surrounding white ring are typical of *Strep. faecium*. This organism partially reduces the TTC when inoculated into the mass of the AGAT medium (pH 7·2–7·4); but no reduction occurs when a streak inoculation is made on to the surface of the TITG medium of Barnes (pH 6·0). Barnes (1956*a*) has already demonstrated the role of pH in the reduction of TTC by *Strep. faecium*. Lenticular colonies with a surrounding white ring are typical of certain *Micrococcus* spp. which are not inhibited in the AGAT medium. However, colonies typical of *Micrococcus* spp. have always been fewer than those of *Strep. bovis* in the samples examined.

Type 3 colonies were red and in the form of a single or double lens (diameter 1–1·5 mm) surrounded by a red zone in the medium where TTC had been reduced. These colonies are typical of streptococci resembling *Strep. faecalis* and have only rarely been observed. Unidentified Gram-positive anaerobic sporeforming bacteria which were partially inhibited in the AGAT medium sometimes formed colonies of this type.

Type 4 colonies were small (diameter less than 0·5 mm), lenticular or irregular red, or small or very small white irregular colonies; these are typical of members of the genus *Lactobacillus* and are partially inhibited, the extent of inhibition depending on the strain, perhaps even the species, of lactobacilli present in the sample.

Colonies of Gram-negative bacteria, aerobic sporeforming bacteria or members of the genus *Corynebacterium* have not been observed on plates of the AGAT medium prepared from dilutions higher than 10^{-3} of the various samples studied during this investigation. Red, irregular, coherent colonies of *Actinomyces* have occasionally developed on plates prepared from the 10^{-2} and 10^{-3} dilutions of gastric samples.

Comparison of AGAT medium with various other selective media for the enumeration of streptococci in a caecal sample and in a pure culture. An example of the results obtained after inoculation of a representative caecal sample into the mass of the AGAT medium and on to the surface of the MEA, TlTG and KF media is given in Table 11. It may be observed that the number of colonies typical of *Strep. bovis* in the AGAT medium was approximately 10 times higher than that in MEA agar and 100 times higher than those on the other two media examined. Although colonies of lactobacilli do not hinder the enumeration of streptococci in the AGAT medium, it is difficult to distinguish between colonies of these organisms in MEA and TlTG. Fewer colonies of lactobacilli developed in the KF medium than in the other two media.

Table 11. *A comparison of the counts obtained with a caecal sample (a) and a pure culture (b) plated on different media selective for streptococci*

Dilution	Counts (/ml) of streptococci and presence of other organisms* in								
	TlTG		KF		MEA		AGAT		GAPTsl
	(a)	(b)†	(a)	(b)†	(a)	(b)‡	(a)	(b)§	(b)§
10^{-7}	0	0	0	0	(L)	7	$1S_1$	7	9
10^{-6}	0	14	0	25	$1S_1$ (L)	62	$7S_1$ (L)	87	115
10^{-5}	$(L+S_1)$		$(L+S_1)$		$(L+S_1)$		$68S_1$ (L)		
10^{-4}	$(L+S_1)$		$(L+S_1)$		$(L+S_1)$		$(L+S_1)$		
10^{-3}	$(L+S_1)$ $11S_2$		$(L+S_1)$ $9S_2$		$(L+S_1)$ $11S_2$		$L+S_1$ $7S_2$		

* Colonies typical of: *Strep. bovis*, S_1; *Strep. faecium*, S_2; lactobacilli, L. Brackets indicate the presence of pin point colonies.

† Colonies pin point in size. ‡ Colonies 0·3 mm in size. § Colonies 1·0–1·2 mm in size.

Because the inhibition of streptococci on the MEA, TlTG and KF media could have been partly because the inoculations had been made on the surface, decimal dilutions of a pure culture of one of the strains of dominant streptococci studied were poured with these three selective media, the AGAT medium and a nonselective medium (GAPTsl). The results obtained on the five media, given in Table 11, show that although the red and rose colonies that developed in the MEA and AGAT media, respectively, were not of the same size, the numbers in the two media were comparable. It may also be seen that pin point colonies developed in the TlTG and KF media and were approximately four times fewer in number than in the AGAT medium, in which the colonies reached a diameter of 1–1·2 mm. However, if the pour plate technique is used for inoculation of a sample from the contents of the alimentary tract of pigs into MEA or TITG, colonies of lactobacilli are too numerous to allow enumeration of streptococci.

Numerical importance of streptococci in the alimentary tract and faeces of pigs. The average, maximum and minimum counts/g of fresh sample obtained in the AGAT medium are given in Table 12. Wide variations may be observed in the counts (average, $9{\cdot}5\times10^4$) obtained from the various samples of gastric contents. The counts obtained from samples of caecal contents and faeces showed less variation, with averages of $3{\cdot}6\times10^7$ and $6{\cdot}9\times10^7$, respectively.

Table 12. *Counts of dominant streptococci obtained in AGAT medium from samples of the alimentary tract of pigs*

Source of samples	No. of samples tested	Log_{10} (count/g of fresh material)		
		Average	Maximum	Minimum
Stomach	27	4·98	6·30	2·00
Caecum	42	7·56	8·70	4·00
Faeces	110	7·87	9·60	4·70

Colonies typical of *Strep. faecium* (Plate 1b) were enumerated on low dilution plates prepared from only 52 of the 179 samples examined. The average count for this species was $3{\cdot}4\times10^5$ However, a count of $1{\cdot}3\times10^7$ was observed in samples from animals receiving a spiramycin supplement to ration A. The average count of *Strep. faecium* in samples from pigs receiving a diet supplemented with the other antibiotics cited by Dickinson & Mocquot (1961) was no higher than that in samples from pigs receiving ration A alone. It is of interest to note that spiramycin was being used for the first time in the piggeries of the C.N.R.Z. during the experiments described in the present work.

4. DISCUSSION

Barnes (1961, pers. comm.) recognizes two types in the species *Strep. bovis* as defined by Orla-Jensen (1919); a type A which is able to synthesize dextrans from sucrose under certain conditions (for example, strain 1 in Table 10), and a type B which is not. Organisms in this species are described as actively hydrolyzing starch and as fermenting lactose, and as fermenting raffinose (Orla-Jensen, 1919) only when they are grown in a casein-peptone medium. However, before *Strep. bovis* was defined as a species, Andrewes & Horder (1906) had isolated from horse faeces, dust, saliva and human urine and faeces numerous lactose nonfermenting strains of streptococci, of which only a few were able to ferment raffinose. These authors proposed the name *Strep. equinus* for their isolates. Winslow & Palmer (1910) compared 302 strains they isolated from 15, 12 and 22 samples of human, horse and cow faeces, respectively. They confirmed that *Strep. equinus*, comprising lactose negative, raffinose negative strains, was the most frequent in horse faeces, whereas they showed that cow faeces contained a more heterogeneous population of streptococci. Hodge & Sherman (1937) adopted the definition of *Strep. equinus* given by Andrewes & Horder (1906) and also noted that "... starch is not hydrolyzed under the conditions of test used. (This test was considered of interest because in some respects *Streptococcus equinus* appears to be related to *Streptococcus bovis* which hydrolyzes starch actively. In three attempts, using both ordinary nutrient agar and yeast-peptone as bases, no growth was obtained on the starch agar plates)." The species *Strep. equinus* has been an object of criticism since that date, for Hodge & Sherman (1937) considered it to be "... a convenient 'waste-basket' into which have been thrown streptococci that do not ferment lactose." This is still the opinion of Seeley & Dain (1960) who said, concerning the two species *Strep. bovis* and *Strep. equinus*, that "... the wisdom of retaining these 2 separate species has come under closer scrutiny in recent years." Ability to hydrolyze starch was given by Seeley & Dain as an additional character of the species *Strep. equinus*. However, Fuller *et al.* (1960) were of the opinion that 74% of the group D strains that they had isolated from pig faeces should be classified in the species *Strep. equinus*, since they did not ferment lactose and did not hydrolyze starch.

From the definitions given above, 34 of the 100 strains studied during this work belong either to the species *Strep. equinus* (13 lactose negative, raffinose negative strains) or to the species *Strep. bovis*, type B (21 lactose positive, raffinose positive strains). The other 66 strains should be considered as variants of *Strep. equinus* (the 64 lactose negative, raffinose positive strains) or as 'unclassified' (the 2 lactose positive, raffinose negative strains). The 100 strains examined have been isolated from a small number of animals of similar age which were housed together and received similar diets, that is to say, under conditions which greatly restricted external variations. It is not, therefore, very satisfactory to separate them into two distinct species and an 'unclassified' type, and to do this on the basis of only two characters. Furthermore, it is even less satisfactory to separate the 29 strains occurring together in the 10^{-7} dilution of a portion of caecal contents into 23 strains of *Strep. equinus*, 5 strains of *Strep. bovis* and 1 'unclassified' strain, or into 17 different types, if fermentation of each single carbohydrate is considered as a character valid for classification. Table 7 shows that in addition certain populations obtained from a single colony (isolated from a high dilution plate), which could have arisen from one or a small number of organisms, may be heterogeneous with regard to their behaviour towards certain carbohydrates, e.g. raffinose, galactose and amygdalin. Wheater (1955) also noted that the *Lactobacillus acidophilus* strains she examined for raffinose fermentation showed "... differences in the results of duplicate tests of the same organism. The differences were, however, comparatively infrequent, occurring in only 1·3% of the tests." Consequently it would appear inadvisable to differentiate the 100 strains of dominant streptococci from the alimentary tract of pigs into two separate species, *Strep. bovis* and *Strep. equinus*, on the single criterion of lactose fermentation.

Andrewes & Horder pointed out the fragility of classifications based on a single fermentation character as early as 1906. Yawger & Sherman (1937) maintained, from a study of lactose negative strains of *Strep. lactis*, that "In a species which shows so many other variations with the fermentation tests, there would appear to be no sound grounds for creating a new species on the basis of the non-fermentation of lactose." Lwoff (1958) has stressed the fact that "Le monde bactérien asexué, où chaque organisme n'aspire qu'à affirmer sa personnalité est l'expression d'un désordre extrême. Il convient d'opposer à cette anarchie en expansion un arbitraire des plus rigoureux. L'appareil de la nomenclature binaire a été forgé à cette fin." In order to conform with these opinions, the authors suggest that the 100 strains they have described should be considered as biotypes within the species *Strep. bovis*, whether or not they ferment lactose and raffinose. Lwoff (1958) has defined biotypes as ". . . groupes d'individus possédant essentiellement le même patrimoine héréditaire et ayant donc nécessairement en commun la grande majorité de leurs caractères." Mieth (1960) also considered lactose nonfermenting strains (51% of the identified strains he had isolated from the faeces of pigs) as variants of *Strep. bovis*. Morélis (1958) concluded that numerous biotypes of the species *Strep. faecalis* also exist. The same problem arises for the separation of species within other genera: for example, Renoux (1958) concluded that the 4,612 strains of *Brucella* studied by various workers are only biotypes of a single species.

However, a character of the streptococci studied during this work should be brought into consideration—their ability to hydrolyze urea. Niven *et al.* (1942) have, in fact, reported that among all the species they studied no strain was able to hydrolyze urea. It does not appear that any group D streptococci capable of hydrolyzing urea have been described since this observation was made, even though it is a character widespread among bacteria. May ability to hydrolyze urea be considered as a differential character valid for use in classification? In the second part of the results presented here it was shown that this character, which is easily demonstrated, is stable after successive subculture of a urease positive strain (258/10) without urea, and it would therefore appear that hydrolysis of urea is catalyzed by a constitutive enzyme system. However, it would be necessary to cultivate this strain in a chemically defined medium in the same manner as Gibbons & Doetsch (1959) in order to substantiate this hypothesis because bacterial action on guanidine compounds with subsequent urea formation in a medium such as LAPTg5 may not be excluded. De Turk (1955) has, in fact, shown that urease production by *Pseudomonas aeruginosa* is adaptive, even though this enzyme appeared in a medium to which urea had not been added. The experiment carried out with strain 248/1 appears to show that, in this case, the ability to hydrolyze urea was acquired. Although the two results obtained in this work appear contradictory, they may in fact be in agreement if it is conceived that all strains possess an adaptive enzyme system, but that certain of them are unable to synthesize the urease inducer in a medium without added urea.

It may be seen from Table 3 that no urease positive strain studied hydrolyzed arginine. However, Mieth (1960) found that a small number of streptococci he had isolated from the faeces of pigs were arginine positive. One of the urease positive streptococci studied during the present work did not acquire the ability to hydrolyze arginine even after several subcultures in the presence of this substrate. It is interesting to note that the strain of *Strep. durans* mentioned did not hydrolyze arginine on first isolation, but acquired this character during the third subculture in a medium containing arginine. Thus both hydrolysis of arginine and urea may be adaptive. However, arginine hydrolysis is employed as a character in the distinction of species of group D streptococci (Niven *et al.* 1942; Abd-el-Malek & Gibson, 1948).

Ability to hydrolyze urea, even though adaptive, does not appear to be acquired by all strains of streptococci. The limited number of experiments carried out here, however, does not permit a definite statement to be made on this point. Similarly, the fact that all the 415 strains studied hydrolyzed urea does not mean that the dominant streptococci present in the alimentary tract of all pigs possess this character. It would be interesting if experiments carried out by other workers could confirm this observation.

Despite, and because of, these restrictions, the authors suggest that the streptococci they have studied should be named *Strep. bovis*, type C, the distinctive character of which would be urea hydrolysis.

Certain differential characters have not been investigated during this work; in particular serological studies have been limited to the reaction with group D antiserum. It is interesting to note that the concentrated extract prepared from one urease positive strain from caecal contents did not react with group D sera although it was identical (in the results of the other 57 tests employed) to certain of the 100 other strains described. Perry, Wilson, Newland & Briggs (1955) have made the same observation with regard to 2 of the 81 strains of streptococci they isolated from the rumen of fistulated cows. It would seem, therefore, that reaction with group D serum may not always be correlated with the physiological and biochemical characters which define the species or group *Strep. bovis*. With respect to the type antigens, Sharpe & Fewins (1960) have shown that several strains of *Strep. faecium* bearing different type antigens could co-exist in one and the same caecal sample from a pig.

It is useful to know how to place the strains of streptococci belonging to the *Strep. bovis* group isolated from the alimentary tract of pigs since they constitute the dominant group present in the intestinal contents of the pigs studied in these and previous experiments (Fuller *et al.* 1960; Mieth, 1960). However, it may be seen from Table 1 that other workers have obtained different results. Kjellander (1960) suggested that both geographical factors and the methods used for enumeration may be responsible for these discrepancies. Medrek & Barnes (1958) reported that only a proportion of strains of *Strep. bovis* can develop well in the TlGT medium and Kjellander (1960) confirmed this fact. Table 11 also shows that although the TlTG medium is excellent for the enumeration of *Strep. faecium*, it does not allow a quantitative enumeration of *Strep. bovis* type C. The AGAT medium devised appears to be better adapted for the selective enumeration of *Strep. bovis* type C than the other three media examined. The use of sodium azide as an inhibitor of Gram-negative organisms and sporeforming strict aerobes, proposed by Mallmann (1940), is now widely practised. Kjellander (1960) has reported that 0·03% of sodium azide is insufficient for inhibiting the development of *Escherichia*, *Proteus* and *Pseudomonas* spp. Gram-negative bacteria are inhibited by a final concentration of 0·03% of sodium azide in the AGAT medium because a period of 'contact' is provided by the inoculation procedure. This technique has been adapted from that used for the study of antiseptics. Sodium glutamate and acridine orange with sodium azide inhibit lactobacilli to an extent sufficient to allow selective enumeration of streptococci. This mixture of glutamate and acridine orange has no adverse effect on the growth of *Strep. bovis* type C, whereas ethyl violet, another inhibitor of lactobacilli proposed by Litsky, Mallmann & Fifield (1953), inhibited 40% of strains belonging to the *Strep. bovis* group (Bartley & Slanetz, 1960). The inhibitory role of certain amino acids for certain lactobacilli has been shown by Teeri & Josselyn (1953), and acridine orange has been employed by Beers, Hendley & Steiner (1958) in a study of the metabolism of polynucleotides by *Escherichia*. The TTC included in the AGAT medium as an indicator of reduction inhibits the development of members of the genus *Corynebacterium* and also partially inhibits *Strep. bovis* type C if the concentration is increased tenfold.

5. SUMMARY

The object of the work reported was to enumerate and identify the dominant streptococci present in the alimentary tract of pigs reared at the C.N.R.Z. A medium (AGAT) containing sodium azide and a mixture of sodium glutamate and acridine orange as inhibitors, and triphenyl tetrazolium chloride as a redox indicator, has been devised. The concentration of inhibitors, coupled with the method of inoculation of the samples, has allowed selective enumeration of streptococci in the presence of a superior number of lactobacilli. Average counts (/g of wet material) of streptococci obtained were $9{\cdot}5 \times 10^4$ in the gastric contents, $3{\cdot}6 \times 10^7$ in the caecal contents and $6{\cdot}9 \times 10^7$ in the faeces. Identification of 100 isolates from high dilution plates of the AGAT medium has been made on the basis of 58 differential characters. All these strains belonged to Lancefield group D and, although they showed the majority of physiological and biochemical characters of *Streptococcus bovis*, strains isolated from the same sample differed in fermentation of lactose and raffinose. The 100 strains described were also able to hydrolyze urea, and it has been shown that the presence of glucose in the medium had an effect on the extent of this hydrolysis.

The name *Strep. bovis* type C has been proposed for the urease positive strains isolated from high dilution plates of AGAT prepared from gastric and caecal contents and faeces of the pigs examined during this work.

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Explanation of Plate

Plate 1. Colonies appearing on plates of the AGAT medium prepared from dilutions of the faeces of a pig (×20).

(a) Three lenticular colonies of streptococci and small or pin point irregular colonies of lactobacilli from a 10^{-6} dilution.

(b) One large lenticular colony typical of *Strep. faecium*, 6 medium lenticular colonies of dominant streptococci, and irregular colonies of lactobacilli from a 10^{-4} dilution.

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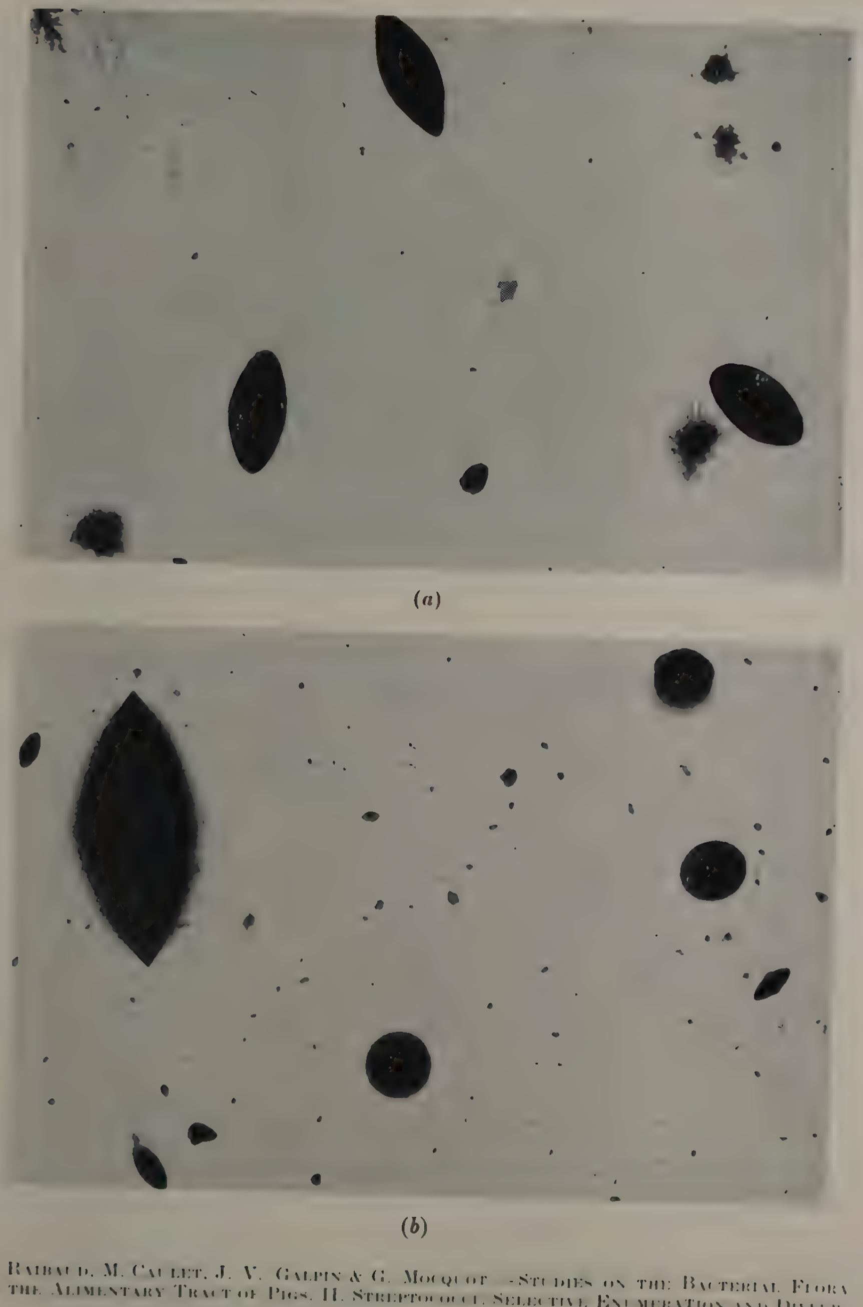

(*a*)

(*b*)

P. RAIBAUD, M. CAULET, J. V. GALPIN & G. MOCQUOT—STUDIES ON THE BACTERIAL FLORA OF THE ALIMENTARY TRACT OF PIGS. II. STREPTOCOCCI. SELECTIVE ENUMERATION AND DIFFERENTIATION OF THE DOMINANT GROUP. PLATE 1

Lev, M. (1961). *J. appl. Bact.* **24** (3), 307–315

(Symposium on Bacteria of the Intestine: Paper V)

GERM FREE ANIMALS AND THEIR USES IN ELUCIDATING THE ACTION OF THE GUT FLORA ON THE HOST

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1. Introduction

The gut flora-host relationship is a complex one, the outcome of which may be beneficial or detrimental for the host. Among the many factors that must be considered when the effect of a particular organism on the host is being studied are: the host itself; the individual organism of particular interest; and the gut flora as a whole (of which the organism forms a part). Germ free animals are a great asset in work of this kind. In recent years they have been used to study the gut flora-host effects, to act as controls to conventional animals and also as a basis into which a defined bacterial flora can be implanted. Germ free animals are animals reared under sterile conditions and it is usual to define their status to avoid unnecessary controversy. The criterion accepted is that growth does not occur when samples of faeces, intestinal contents, organs, etc., are inoculated on media designed to grow known cultivable bacteria and fungi.

2. The Design and Use of Modern Apparatus

The design of germ free rearing apparatus is based on a simple concept, namely, a compartment which can be sterilized and is capable of housing animals. Provision must also be made for supplying sterile air, food and water. In order to obtain a workable design, stringent precautions must be followed to ensure sterility: the apparatus must be gas tight; the adequacy of sterilizing procedures for diets must be ascertained; and the mode of entry and amount of materials must be carefully checked.

Steam is the method of choice for sterilizing germ free units and the design of the apparatus has been influenced by this consideration. Two main types are in use: the autoclave type and those which can be autoclaved. Reyniers and his colleagues at the Lobund Institute have been working with germ free animals for the past 30 years and have designed a unit which consists essentially of a stainless steel autoclave to which is attached a smaller autoclave (Reyniers, Trexler & Ervin, 1946; Reyniers, 1959). The smaller autoclave serves as a lock through which food is passed, and samples and refuse removed. It can also be fitted with a germicidal trap for entry of heat labile materials. Air is sterilized by filtration through glass wool. This apparatus has been used extensively in the United States and most of the experimental results available have been obtained with it. Miyakawa (1959) has used a similar design fitted with a remote control system with which he can handle animals. Both units are very expensive.

The second type of germ free unit is made of light gauge materials which can be autoclaved. Gustafsson (1948) has designed an elegant piece of apparatus consisting of a stainless steel box fitted with a plate glass top (Plate 1). Air is sterilized by incineration at 300° and is cooled before entry into the unit. Access to the chamber is made via a germicidal trap.

A plastic unit made of nylon which can be autoclaved has been used for chicks at the National Institute for Research in Dairying (Lev, unpublished data). This was designed for maximum simplicity and as a prototype to be used with mammals. It consists of a nylon tube supported by a frame, part of the tube being constricted to form the germicidal trap (Plate 2, a and b). Plastic units of another type are available commercially in the United States (Trexler & Reynolds, 1957), but these suffer from the disadvantage of not withstanding autoclaving and thus require chemical disinfection by peracetic acid or β-propiolactone.

Chicks, turkeys, guinea pigs, rats and mice have all been used in experimental germ free research, and other animals such as monkeys and pigs have also been reared under germ free conditions.

To obtain germ free chicks, eggs are taken at the 18th day of incubation. The shells are sterilized by washing in detergent and by immersion in mercuric chloride solution. The eggs are then introduced into a sterile germ free unit via a germicidal trap and allowed to hatch.

For obtaining first generation germ free mammals in the Reyniers system, the procedure is as follows: a pregnant female is taken just prior to delivery and the abdomen shaved and sterilized with iodine solution. The animal is placed on its back on a platform which when raised brings the abdomen into contact with a sheet of plastic. The plastic forms part of a specially designed aseptic operating unit in which two people can work. By cutting through the plastic and body wall from above using a cautery, the uterus can be removed, and the young animals released. They are then handed to an assistant who cleans and revives them. Guinea-pigs are able to walk about and feed shortly after delivery, whereas rats and mice have to be hand fed at regular intervals—a very exacting procedure. Urination and defaecation must be stimulated as these processes do not occur spontaneously in rats and mice.

The greatest hazard in this work is bacterial contamination. As soon as the animals are established in their germ free units, and at regular intervals thereafter, they are tested for the presence of contaminants. Samples of faeces and anal swabs are placed in liquid and solid media designed to grow aerobes, anaerobes and fungi; additional samples are examined for the presence of pleuropneumonia-like organisms. Smears are also made from anal swabs and examined microscopically. Contamination of the germ free units is also indicated by the occurrence of a foul odour.

Another factor which plagues germ free animal research is nutritional deficiency, which may be caused by the destruction of vitamins during sterilization of the diet and which may be accentuated by the absence of a gut flora. The considerably enlarged caeca found in germ free mammals (Wynngate, Horton & Forbes, 1958) appears to be due only to the absence of bacteria. The caecum of the germ free guinea-pig does not appear to evacuate and the caecal wall becomes stretched very thin.

The factors outlined above are a few of the many which can cause failure in experiments.

3. Applications of Germ Free Animals

(a) *Growth stimulation by antibiotics*

The mode of action of growth stimulation by antibiotics has been the subject of intense study and still remains an intriguing problem. It is well known that when small amounts of certain antibiotics—penicillin, streptomycin, aureomycin, etc.—are fed to young animals, their growth rate is increased. That this increase was associated with the bacterial flora of the gut was anticipated at the outset, yet the literature on this subject illustrates the confusion which exists, as almost every species of intestinal organism has been implicated. In the type of experiment usually followed the numbers of bacteria in the gut or faeces of animals supplied with and not given antibiotics in the diet are compared and the observed differences correlated with increases in growth rate. The results can often be interpreted in various ways, however; thus an increase in the numbers of lactobacilli may either be beneficial to the animal, since these organisms may depress others capable of producing toxins, etc., or detrimental, because they are, on the whole, nutritionally fastidious organisms which may compete with the host for nutrients. Hypotheses were put forward to fit the experimental contingency but without *direct* evidence that the observed differences in numbers of bacteria between the antibiotic fed and unsupplemented groups had had any physiological effect on the host.

In studies with chicks at the National Institute for Research in Dairying it was shown by Coates *et al.* (1952) that environment played a large part in this growth stimulating effect and that it was due not to a growth stimulation *per se* but to a relief (by the antibiotic) of depression of growth. Chicks kept in old premises showed a response to antibiotics, whereas chicks kept in new premises did not. The chicks in new quarters grew at the same rate as those fed with antibiotic (in this case penicillin) in the old quarters. When the chicks in new quarters were fed with faeces from the chicks in the old premises they grew poorly and responded to penicillin. Thus the growth depression was transmissible.

When the bacterial floras of the antibiotic supplemented and unsupplemented groups were compared, the only consistent difference noted was the presence or absence of *Clostridium welchii* (Lev, Briggs & Coates, 1957). *Cl. welchii* established itself very quickly in the caeca of the chicks kept in the old quarters and was eliminated by the penicillin in the diet (Plate 3, a and b). The organism appeared later in the gut of chicks in the new premises. Chicks were examined from alternative sources and in some cases penicillin did not reduce the numbers of *Cl. welchii*. The counts of *Cl. welchii* from the caeca of chicks fed with and not given penicillin are shown in Plate 3, c and d. The numbers in the two groups here are almost identical, but the lecithinase (α toxin) production on neomycin-egg yolk-serum agar was markedly reduced with organisms from the penicillin group. The physiological effect on the host of this reduction in toxigenicity would be the same as elimination of the organism.

At this stage germ free animal techniques seemed to permit a direct approach to the problem. It had been shown at both the Walter Reed Army Institute of Research and the Lobund Institute, that germ free animals did not respond to antibiotics. At the Walter Reed Institute an attempt was made in co-operation with Dr. Martin Forbes to determine whether *Cl. welchii* had any real physiological effect or whether it was acting merely as an 'indicator organism' and had no relation to the problem (Lev & Forbes, 1959). Accordingly, spores of a strain of *Cl. welchii* isolated in the previous experiments were fed to germ free chicks, and this caused a growth depression which was relieved in most part by adding penicillin to the diet (Table 1). The organism was completely eliminated by the small amount (45 p/m) of antibiotic supplied. Since animals with only one species of organism in the gut do not occur in nature it was decided to test a model bacterial flora implanted in germ free chicks. This was composed of a representative of each of the three major groups of bacteria in the chick gut—a *Lactobacillus lactis*, a *Streptococcus liquefaciens* and an *Escherichia coli*, all of which had been isolated from chicks kept in the old quarters in England and shown to have no effect on the growth rate of the chick, but in this system penicillin had no stimulating effect (Table 2).

Table 1. *The growth and growth responses to dietary procaine penicillin G of germ free chicks and chicks with an intestinal flora composed solely of* Cl. welchii *type A*

(From Lev & Forbes, 1959)

Intestinal flora	Mean wt* (g) of chicks given		Difference (*b*–*a*) (g)	Significance of difference‡	
	No supplement (*a*)	Penicillin† (*b*)		t	*P*
None	257	254	−3	1·00	NS
Cl. welchii	213	243	+30	2·66	<0·05

* Results obtained on the 21st day from two replicate experiments each with 14 chicks/treatment. † Included at the rate of 45 mg/kg of diet. ‡ NS, not significant. The difference between the germ free chicks and those given *Cl. welchii*, all on the unsupplemented diet, was highly significant (t = 3·69, *P* <0·01).

Table 2. *The growth and growth responses to dietary procaine penicillin G of germ free chicks and chicks with a defined intestinal flora*

(From Lev & Forbes, 1959)

Intestinal flora	Mean wt.* (g) of chicks given		Difference (*b*–*a*) (g)	Significance of difference‡	
	No supplement (*a*)	Pencilliin† (*b*)		t	*P*
None	240	241	+1	1	NS
Three species of bacteria§	240	232	−8	1	NS
Three species§ plus *Cl. welchii*	213	228	+15	1·72	0·1

* Results obtained on the 21st day from two replicate experiments each with 18–20 chicks/treatment. † Included at the rate of 45 mg/kg of diet. ‡ NS, not significant. The difference between chicks with 3 species of bacteria and chicks with 3 species and *Cl. welchii*, all on the unsupplemented diet, was highly significant (t=2·8, *P* <0·01).

The addition of *Cl. welchii* to this flora produced a growth depression which was less marked than that which occurred when *Cl. welchii* was the sole organism in the gut. This depression was also relieved in most part by adding penicillin to the diet. It is of interest that in one of these experiments penicillin had little effect on the number of *Cl. welchii*. However, as observed in the previous experiments, the toxigenicity of these organisms was reduced.

It is notable that the guts of chicks fed antibiotics are thinner than those of birds on an unsupplemented diet (Coates, Davies & Kon, 1955), and it may be remarked that the three toxins—α, K and θ—produced by these strains of *Cl. welchii* are lecithinase, collagenase and haemolysin, respectively, and may be expected to irritate the gut and cause it to thicken, with resulting impaired absorption of nutrients and hence a slower growth rate. Of interest in this connection is the observation by Wostman, Wagner & Gordon (1960) that thickening of the gut occurred in germ free chicks implanted with *Cl. welchii* and that the feeding of penicillin prevented the thickening.

These studies are being extended at the National Institute for Research in Dairying. Experiments are being done to determine which toxin depresses growth when fed to germ free chicks and the effect of the toxin on the intestinal mucosa of the germ free animal.

(b) *Vitamin requirements of the host*

The contributions of the gut flora to the vitamin requirements of the host animal have been analyzed using germ free animals. Vitamin A deficiency terminates in gross infection and death in conventional animals and has been termed the 'anti-infective vitamin'. The alteration in the mucous membranes due to the deficiency may increase the infectivity of the gut flora and it was of interest, therefore, to determine whether the bacteria were contributing to the death of the host animal. Germ free animals would also give a clearer picture of the lesions formed. Beaver (1961) kept germ free rats on a diet deficient in vitamin A and found that germ free and conventional animals both showed the same survival pattern. Germ free rats showed lesions of the kidney and liver which were not found in conventional animals.

Thus the bacteria of the animal played no part in death due to vitamin A deficiency.

Gustafsson (1959) has made some interesting studies on the utilization of several compounds of the vitamin K group by germ free rats. Bacteria synthesize mainly the vitamin K_2 series and plant material supplies vitamin K_1. However, several synthetic forms, such as menadione (vitamin K_3) are used in diets. Gustafsson found that germ free rats fed a diet deficient in vitamins of the K group showed a prolongation of blood clotting time compared with conventional rats fed the same diet. This is to be expected, as the bacterial flora of the gut contributes to the vitamin K supply of the host. However, menadione and other synthetic forms (sodium menadione sulphate and 2:3-dimethyl-1:4-naphthohydroquinone) neither reversed the K deficiency nor restored the blood clotting mechanism. Besides vitamin K_1 only menadione phosphate had this ability, although less efficiently. Since the synthetic forms of vitamin K are efficacious in conventional animals, it is interesting to speculate that bacteria in the gut can convert menadione into the full vitamin which is utilizable by the host.

(c) *Microbial interrelationships as factors in pathogenicity*

Germ free animals have been used with advantage in the study of microbial interrelationships as factors in pathogenicity. Examples of applications in which a component of the normal gut flora is necessary to establish an infection will be discussed, and an example given of the inhibition of an infecting organism by a member of the gut flora.

Phillips & Wolfe (1959) have studied the relationships between bacteria from the gut flora of animals and the pathogenesis of *Entamoeba histolytica* in amoebiasis. An association with bacteria was suspected because the disappearance of amoebae from the intestine was sometimes observed when bactericidal, but not amoebicidal, agents were fed.

Germ free guinea pigs were inoculated intracaecally with amoebae, but none of these animals exhibited lesions at post mortem. There appeared to be no multiplication of the amoebae in the lumen of the germ free intestine and the organisms survived for a few days only. However, when amoebae were inoculated into germ free guinea pigs previously fed with and harbouring *E. coli* or *Aerobacter aerogenes*, lesions of the caeca developed. The nature of the bacterial factor which enables the amoebae to attack the intestinal wall is not clear. Phillips & Wolfe (1959) fed autoclaved caecal contents of conventional guinea pigs to germ free guinea pigs and observed a reduced infection compared to the bacteria-amoeba challenge. Tests with filtrates of caecal contents of conventional guinea-pigs produced a low incidence of infection. Lowering the E_h of the gut (and there is a marked difference between the E_h of the gut of conventional and germ free guinea pigs) also resulted in approximately half the animals being infected. The factors therefore appear to be complex. However, these experiments demonstrate that organisms, which in themselves are harmless to the host, can produce a lethal infection in association.

Formal & Forbes (1958) have used germ free guinea pigs to study the antagonism of *E. coli* to *Shigella flexneri* infection. The latter does not cause a disease in conventional guinea pigs but killed germ free animals 48 hr after inoculation. However, when a strain of *E. coli* had been administered before the challenge with *Shig. flexneri* the animals survived, and when the intestinal contents were examined 48 hr after inoculation no shigellas were recovered. This strain of *E. coli* did not produce colicine, and antagonism to *Shig. flexneri* could not be demonstrated *in vitro*. An understanding of the mechanism of this inhibition would be of great help in the study of population levels in the gut of animals.

(d) *Breakdown in the defence system against bacteria: haemorrhagic shock*

An example of the toxic effect of the gut flora (i.e. a breakdown of the defence system against bacteria) is seen during the production of acute haemorrhage and is known as irreversible haemorrhagic shock. If a certain blood volume is removed rapidly from a dog, rabbit or rat and sufficient time allowed to elapse irreversible shock ensues terminating in death. One of the major symptoms is a massive haemorrhage into the small intestine. The mechanism of this phenomenon has been extensively studied at Boston by Fine, Ruttenburg & Schweinburg (1959), who showed that endotoxin emanating from Gram-negative organisms in the gut is responsible for toxic manifestations. Endotoxin was demonstrated in the blood of shocked animals and this blood was lethal for animals subjected to reversible haemorrhagic shock. Also, animals in shock are much more susceptible to the action of endotoxin. There is also much further evidence to support the endotoxin hypothesis.

At first sight it appeared that the question of bacterial participation or non-participation in haemorrhagic shock might be readily answered by germ free techniques. Zweifach, Gordon, Wagner & Reyniers (1958) bled germ free rats and found no differences in the production of shock and its manifestations between these and conventional animals. Germ free animals showed the same intestinal haemorrhage. These results were confirmed by McNulty (1958). However, the diet which the germ free animals were fed contained bacteria, and dead bacteria are common in smears of faeces of germ free animals. These bacteria may supply the minute amount of endotoxin required to produce irreversible shock. This problem could be resolved if germ free rats were to be reared on the synthetic amino acid diet of Greenstein, Birnbaum, Winitz & Otey (1957), which could be obtained endotoxin free. Conventional rats have been reared on this diet and the production of germ free animals on a synthetic diet would have applications reaching into the nutritional as well as the physiological fields.

4. DISCUSSION

It can be seen from the examples mentioned above that the judicious use of germ free animals can give answers to problems of gut flora-host relationships which could not be obtained by other means. By enabling individual bacterial-host systems to be studied in isolation it can be shown which bacteria from the complex components of the gut flora are physiologically active. However, there is still a vast amount of work to be done on germ free animals themselves; the unknown cause of the enlarged caecum in germ free mammals exemplifies our lack of knowledge of the physiology of life without bacteria. Germ free animals, therefore, cannot be regarded solely as means for obtaining a ready answer to these problems. They must themthemselves be studied in order to facilitate our understanding of the influence of bacteria on the host.

5. SUMMARY

Several types of apparatus are available for obtaining and rearing germ free animals. Some applications of these animals are discussed. In the study of the antibiotic growth stimulation of chicks, *Clostridium welchii* depressed the growth rate and penicillin reversed this depression. This effect also occurred in the presence of a model bacterial flora. The germ free state had no influence on the symptoms of vitamin A deficiency; however, germ free, as opposed to conventional, rats could not utilize several synthetic forms of vitamin K. *Entamoeba histolytica* required the presence of *Escherichia coli* to produce lesions in the germ free guinea pig, whereas the pre-establishment of *E. coli* in these animals prevented superinfection with *Shigella flexneri*. The study of bacterial participation in irreversible haemorrhagic shock by germ free animal techniques is also discussed. Germ free and conventional animals showed the same pattern of shock. The possibility exists that a small amount of toxin in dead bacteria in the diet of germ free animals may cause shock after haemorrhage.

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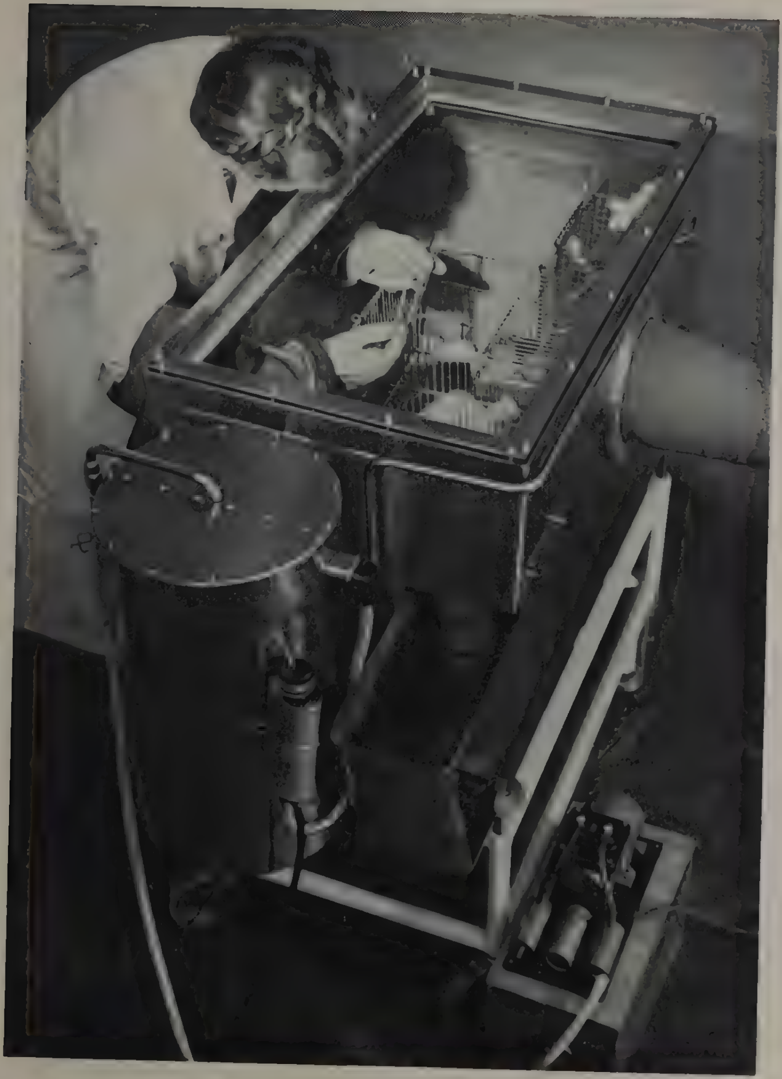
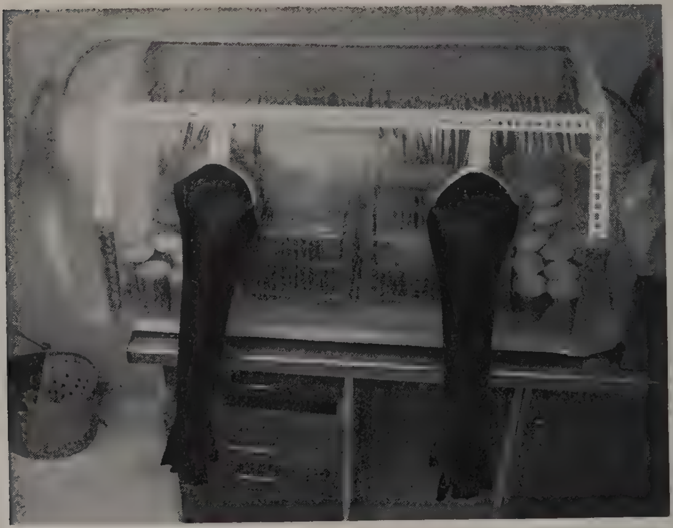


M. LEV — GERM FREE ANIMALS AND THEIR USES IN ELUCIDATING THE ACTION OF THE GUT FLORA ON THE HOST. PLATE 1



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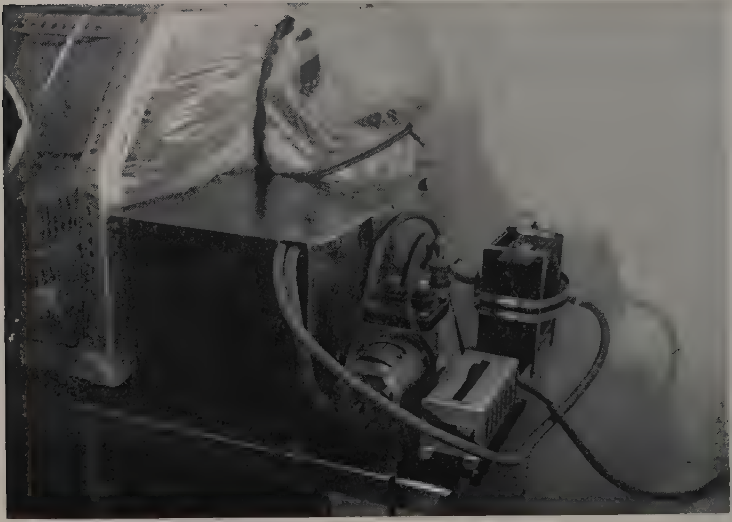

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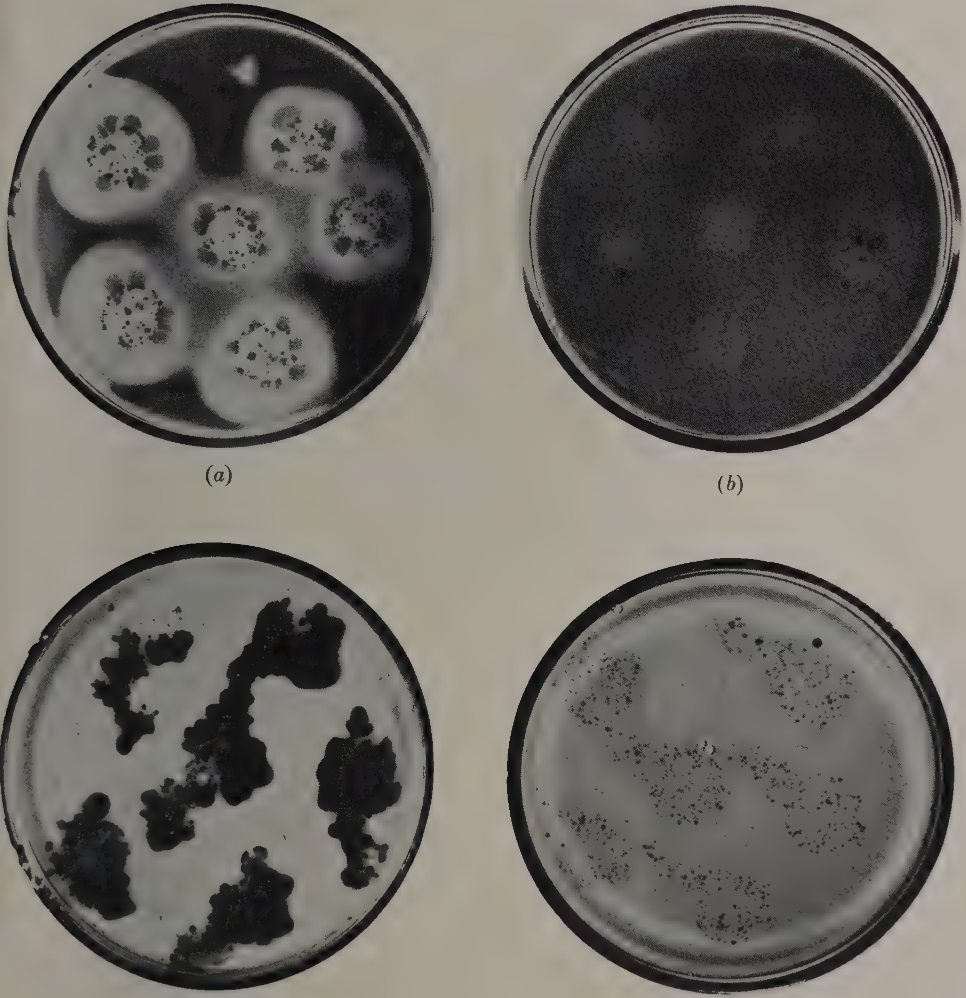
M. Lev — Germ Free Animals and Their Uses in Elucidating the Action of the Gut Flora on the Host. Plate 2



(*a*) (*b*)

(*c*) (*d*)

M. LEV — GERM FREE ANIMALS AND THEIR USES IN ELUCIDATING THE ACTION OF THE GUT FLORA ON THE HOST. PLATE 3

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Explanation of Plates

Plate 1. The Gustafsson germ free unit.

Plate 2. An autoclave plastic germ free unit.
- (a) General view.
- (b) End view showing the germicidal trap, air pump and filter.

Plate 3. Cultures of organisms from the caeca of chicks. ($\times \frac{5}{8}$). (From Lev & Forbes, 1959).
- (a), (b) Differences in spore population of the caeca of chicks 1 day after feeding. Haemolytic colonies present on blood plate inoculated with material from infected chicks (a) are not present on plate inoculated with material from infected chicks receiving penicillin (b).
- (c) and (d) Differences in lecithinase production on neomycin-egg yolk agar between *Cl. welchii* from chicks reared in old quarters (c) and chicks receiving penicillin (d).

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(Symposium on Bacteria of the Intestine: Paper VI)

HOST SPECIFICITY AND ENTEROPATHOGENICITY OF *ESCHERICHIA COLI*

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1. Introduction

For many years certain observers believed that *Escherichia coli* caused disease of the intestinal tract, but this belief has always been criticized on the grounds that these organisms are always present as normal inhabitants of the gut of man and animals. Colonization of the infant gut with *E. coli* occurs soon after birth and the same would appear to be true of many animal species. It has been suggested that the infant gut is colonized by *E. coli* from the mother, but this aspect has not been studied using serological methods of identification.

2. The Serological Classification of *E. coli*

The investigation of enteritis associated with *E. coli* became possible as the result of the work of Kauffmann (1943, 1954), Knipschildt (1945), Vahlne (1945) and

Ørskov (1951), who developed a serological method for the identification of this group which is accepted internationally. This classification has made possible the comparison of findings in different parts of the world as well as comparisons between the serotypes isolated from man, animals and other sources.

The serological classification of *E. coli* depends on the identification of the somatic (O), the surface (K) and the flagellar (H) antigens to which are assigned numerals; for example the antigenic structure of one serotype is written as O1.K1.H7. In this review particular attention will be given to the distribution of the *E. coli* O groups, as by comparison much less is known about the distribution of K and H antigens.

3. THE OCCURRENCE OF *E. COLI* SEROTYPES IN HUMAN SOURCES

(a) *In infantile diarrhoea*

Serological methods for the identification of *E. coli* were used by Goldschmidt (1933) in studies of infantile diarrhoea, and by Lovell & Hughes (1935) for investigating calf scours, but the importance of *E. coli* was not fully appreciated until Bray (1945) investigated outbreaks of infantile diarrhoea and showed that a particular serotype of *E. coli* was isolated from the affected babies; later Giles & Sangster (1948), Taylor, Powell & Wright (1949) and Kauffmann & Dupont (1950) not only provided similar evidence but, as was later demonstrated, the serotype isolated by Bray was shown to be the same as many of the strains isolated in subsequent studies, a type now known as O111.K58.

Since these early studies groups O26 and O55 have been found to be associated with infantile diarrhoea in all countries in which this problem has been studied: Taylor & Charter (1952) and Smith (1955) in the United Kingdom; Braun & Specht (1955) in Germany; Buttiaux, Nicolle, Le Minor & Gaudier (1956) in France; Ewing, Tatum & Davis (1957) in the United States; Mushin, Lowson & Bishop (1960) in Australia; Lie Kian Joe *et al.* (1960) in Indonesia; and Bhat & Myers (1961) in India, to mention but a few. At various times additional serotypes have been implicated, of which the most important are O86 (Braun & Resemann, 1952), O128 (Taylor & Charter, 1955, and Rogers, Kracknell, Buchanan & Halley, 1955), O119 (Smith, 1953) and O127 (Ewing, Tanner & Tatum, 1955). Additional types have been isolated from patients with infantile diarrhoea but their aetiological role is doubtful (see Table 1). In many countries all or most of the serotypes listed above

Table 1. *The O serotypes of* E. coli *isolated from babies with infantile diarrhoea* (A) *and from healthy babies* (B)

A	18, 20, 25, 26*, 28, 44, 55*, 86, 111*, 112, 114, 119*, 124, 125, 126, 127*, 128*	B	1, 4, 6

* The most important types.

are accepted as the cause of infantile diarrhoea, mainly because epidemiological data have supported this interpretation.

Experimental studies have been reported by many workers. Strains isolated from sick babies were fed to volunteers by Kirby, Hall & Coackley (1950), Neter & Shumway (1950), Ferguson & June (1952) and June, Ferguson & Worfel (1953), with the result that diarrhoea was produced in the majority of the volunteers. The duodenum and jejunum of the healthy baby and adult does not contain *E. coli* and is usually sterile, but pathogenic *E. coli* are present in this area in babies with infantile diarrhoea (Thomson, 1955). Koya, Kosakai & Fukasawa (1954), in experiments using adult volunteers, found that enteritis developed in those in which material taken from the duodenum showed the proliferation of *E. coli*. Strains from healthy babies were without effect.

(b) *In healthy persons*

So far a brief review has been given of the studies supporting the view that certain serotypes of *E. coli* cause infantile diarrhoea. It is fair to say that the opposite view is held by many who believe that these particular serotypes may be the result of diarrhoea caused by another agent which may favour the growth of these serotypes, or that at most these serotypes are important only as secondary invaders of the intestinal tract. The arguments are that *E. coli* is the normal inhabitant of the intestinal tract of man and animals and that these serotypes appear to be the same as nonpathogenic types in their behaviour under laboratory conditions, such as biochemical tests and growth at 44°. It is true that in outbreaks of infantile diarrhoea many of the contacts have been found to excrete the epidemic type and yet to remain symptomless.

Payne & Cook (1950) and Gamble & Rowson (1957) isolated enteropathogenic *E. coli* serotypes from children who remained healthy; Ørskov, Ørskov & Paerregaard (1956), in a careful study of babies under one year of age, concluded that the same serotypes occurred with the same frequency in healthy and sick babies. A recent study by Solomon, Weinstein & Sumner (1961) showed a carrier rate of 4·7% of enteropathogenic serotypes and these observers stated that they believed that *E. coli* was not a cause of diarrhoea.

So far pathogenic serotypes concerned with infantile diarrhoea have been discussed. There is little information about the distribution of nonpathogenic serotypes, but Gage, Gunther & Spaulding (1961) showed that serotypes O1, O4 and O6 were commonly found in the faeces of healthy babies. Kauffmann & Perch (1943) and Perch (1944) studied the serotypes of *E. coli* in the faeces of two persons at intervals over a period of six months and found they were able to identify many types; they came to the conclusion that whereas some types were isolated a few times others were present for long periods. This finding agrees with that of Sears, Brownlee & Uchiyama (1950), who called these types 'transients' and 'residents', respectively.

This review shows that serotypes from human faeces have been studied to determine their relationship to diarrhoea. In addition, a few studies have been undertaken to determine the distribution of the enteropathogenic and other serotypes in healthy people. These studies are not sufficiently advanced for it to be said whether an animal, food or other material has been contaminated by *E. coli* of human origin, although strains of *E. coli* O111, O127 and O128 would be suggestive of human contamination as these are common in infantile diarrhoea and have been isolated from adults but rarely from other sources.

4. *E. COLI* SEROTYPES IN DISEASED AND HEALTHY ANIMALS

(a) *Calves*

For many years *E. coli* has been known to be associated with white scours in calves. As far back as 1893 Jensen showed that the condition could be reproduced by feeding calves with *E. coli* which had been isolated from animals suffering from this disease. More recently strains from sporadic cases and outbreaks have been identified by serological methods. This work was started by Wramby (1948) in Sweden, and later Bokhari & Ørskov (1952) and Ørskov (1952) grouped many of Wramby's strains according to the international classification and showed that the most common group was O78, followed by O15, O8, O9, O45 and O26, the last being the only type common to both human and calf diseases. Lovell (1955), in his review, showed that in England O26 and O114 were isolated from calf scours, these being groups that were also isolated from cases of infantile diarrhoea. Nevertheless, O78 and O9, groups common in Sweden, were also common in England. Rees (1958*a*) confirmed the preponderance of O78 in a study of sporadic cases and also isolated two strains of O86, a type found in infantile diarrhoea particularly in Germany. Wilssens & Buttiaux (1958) in a study of 37 cows failed to isolate any strains associated with human disease. Referring to calves once more, Glantz, Dunne, Heist & Hokanson (1959), working in the U.S.A., reported the identification of O78 but found O8 was most common with O119 second in incidence in their series. Fey (1956, 1957) showed clearly the association of O78 and calf scours; he rarely isolated this type from normal calves or cows or from human material in Switzerland. The prevalence of O78 in cases of calf scours was reported in Denmark also (Dam, 1960). From these reports it is clear that O78 occurs in many parts of the world and is directly related to calf scours; nevertheless there is still some controversy as to whether *E. coli* is a true aetiological agent (Smith, 1960). Table 2 shows the serotypes which have been

Table 2. *The O serotypes of* E. coli *isolated from animals*

Source	Serotype
Calf scours	1, 3, 4, 7, 8, 9, 11, 15*, 17, 18, 20, 21, 23, 26*, 35, 41, 45, 78*, 82, 86, 88, 89, 103, 107, 113, 114, 115, 116, 117, 126, 137
Swine oedema	5, 8, 46, 75, 138*, 139*, 141
Swine enteritis	8*, 138, 141*
Lamb disease	24, 78*
Hen septicaemia	2
Healthy hens	26, 55, 119, 128

* The most important types.

isolated from calf scours, though the aetiological role of most of these types is unproven.

(b) *Pigs*

In pigs two diseases of the intestine, oedema disease and swine enteritis, are associated with certain serotypes of *E. coli*. Ewing, Tatum & Davis (1958), working with strains isolated from cases of oedema disease in the United States and Ireland, found O groups 138 and 139 to be the most common in strains from both countries; these were also isolated in England by Sojka, Lloyd & Sweeney (1960), who also found that types O141, O8 and O138 were more usually associated with swine enteritis. In Canada, Kelen, Campbell & Barnum (1959) found that O141, O138 and O139 were the predominant types in disease. A number of additional serotypes have been isolated from cases of pig diseases (Table 2). The characteristic property of *E. coli* strains associated with pig diseases is their ability to cause haemolysis when grown on blood agar plates; this character is rarely found in strains associated with intestinal disease of man or other animals.

(c) *Lambs*

In Australia, Roberts (1958) described an outbreak in lambs in which the symptoms were similar to calf scours; from the lambs examined *E. coli* O78 was isolated. In England, Rees (1958*b*) also isolated O78 and O24 from scouring lambs.

(d) *Hens*

Edwards & Ewing (1954) investigated 30 strains of *E. coli* isolated from the organs of fowls, many of which had suffered from septicaemia; all these strains belonged to type O2. These workers failed to isolate type O2 from the intestines of fowls, other domestic animals or human material. Thomson (1956) cultured the droppings of healthy chickens in a search for a reservoir of serotypes related to infantile diarrhoea and found the following O serotypes: 26, 55, 119 and 128.

(e) *Cats and dogs*

Mian (1959), working in the U.S.A., examined the faeces of healthy dogs and cats for five *E. coli* serotypes associated with infantile diarrhoea; he found types O111, O55 and O26 in 12% of dogs and 8% of cats. Mackel, Weaver, Langley & De Capito (1960) investigated kittens and isolated the human types O55, O114, O126 and O128 (Table 3).

Table 3. *The O serotypes of* E. coli *isolated from healthy household pets*

Source	Serotypes
Dogs	26, 55, 111
Cats and kittens	26, 55, 111, 114, 126, 128

(f) *Monkeys*

Ewing, Galton & Tanner (1955) reported the isolation from monkeys of serotype O111, the detailed antigenic structure of which differed from the human type used for comparison. It is possible, nevertheless, that some human types may be found to be identical with the monkey types.

5. EXPERIMENTAL INVESTIGATIONS USING ANIMALS

The distribution of *E. coli* serotypes has been investigated to determine their relationship to certain diseases in man and animals. When such a relationship was found to exist a search was made for reservoirs of the important types. The types which cause infantile diarrhoea have been isolated from a number of animal species (Table 4). It is true that occasionally there was some difference in the detailed structure of the types isolated (for example, a human type was O111 ab, whereas the type isolated from monkeys was O111 ac), but rarely have serotypes been investigated in such detail, and it may be that types isolated in one country may differ from similar types in another in their detailed structure. Similarly, when considering the flagellar antigens in conjunction with the somatic, then certain flagellar types are more common in human disease: for example O111.K58.H2 has been described on many occasions in infantile diarrhoea, but has rarely been isolated from other sources. Because of the difficulty of interpreting the data on *E. coli* serotypes and their relationship to diseases in man and animals, much experimental work has been done using human volunteers (which was mentioned earlier) and laboratory animals.

Table 4. *The sources of the important serotypes of* E. coli

Serotype	Main sources	Subsequent sources
O26	Infantile diarrhoea	Calf scours, bovine mastitis, milk, piglet enteritis, chicken faeces, dogs, cats, kittens
O55	Infantile diarrhoea	Calf scours, bovine mastitis, milk, bull semen, chicken faeces, dogs, cats, kittens
O111	Infantile diarrhoea	Bovine mastitis, piglet enteritis, chicken faeces, dogs, cats, kittens
O119	Infantile diarrhoea	Cow's milk, chicken faeces
O127	Infantile diarrhoea	
O128	Infantile diarrhoea	Cow's milk, chicken faeces, cats, kittens
O78	Calf scours	Lamb disease
O8 O138 O139 O141	Swine diseases	

(a) *Method*

A method described by Violle & Crendiropoulo (1915) and rediscovered by De & Chatterje (1953) appeared to show the ability of an organism to cause diarrhoea.

A rabbit was allowed to drink water and was starved for 24 hr. It was then anaesthetized and an abdominal incision made exposing the small intestine. A 4 in. loop was ligated at each end, 1 ml of a suspension of the test organism injected into the ligated loop, the abdominal incision closed and the rabbit kept without food or water for a further 24 hr, when it was sacrificed, a post mortem performed and the intestine examined. Cultures were made of the contents of the ligated loop and histological sections made of the gut wall.

(b) *Results*

In their studies De, Bhattacharya & Sarkar (1956) and Taylor, Maltby & Payne (1958) found that *E. coli* serotypes isolated from cases of infantile diarrhoea caused dilatation of the ligated loop (which was due to a mucopurulent exudate) whereas *E. coli* types from healthy babies were without effect. The number of organisms in a positive loop was the same as in a negative loop, so that the effect was not due to a failure of multiplication. In later studies Taylor, Wilkins & Payne (1961) found that whereas serotypes such as O111.K58 isolated from a case of infantile diarrhoea caused dilatation of the ligated loop, strains of the same somatic type isolated from a healthy child or from water were without effect; the conclusion was that strains causing infantile diarrhoea produced a toxic substance which also caused the mucopurulent exudate in the ligated gut. Strains from animal disease were also tested: some calf scour strains caused dilatation, some failed to do so. No positive results were obtained with strains from swine oedema or swine enteritis. Strains of *E. coli* associated with swine oedema are haemolytic and, as already stated, belong to certain serological types. Intravenous inoculation of cultures by Gregory (1955) or extracts by Erskine, Sojka & Lloyd (1957) and Timoney (1957) in pigs caused a similar disease, but Gitter & Lloyd (1957) failed to confirm these results. An extract and a strain of *E. coli* which had been tested in pigs and found to cause oedema disease by Timoney were tested in the ligated rabbit gut, but neither the living culture nor the extract produced dilatation.

6. DISCUSSION

Diseases of the intestine caused by *E. coli* fall into two categories: those in which the infection is localized in the gut; and those associated with the spread of infection to other parts of the body. The ability of *E. coli* to cause intestinal disease was reviewed by Taylor (1959). In infantile diarrhoea *E. coli* infection is localized in the intestine and there is no invasion of other parts of the body; the infecting organism can be isolated from all parts of the baby's large and small intestine including the jejunum and duodenum, the two last mentioned parts being sterile in the healthy subject. The strains of *E. coli* concerned with infantile diarrhoea have the ability to cause dilatation of the ligated rabbit gut; this characteristic may determine their classification as pathogenic bacteria. The serotypes having this characteristic which have been isolated from human infection have not as yet been isolated from animals or from other sources; they appear to be host specific. Similar serotypes have been isolated from water, hens and other animals, but those tested have failed to affect the ligated rabbit gut. In calf scours and some other animal diseases there are many reports of a spread of the organism to the blood stream resulting in septicaemia, so it is possible that the ability of the human enteropathogenic strains to cause dilatation of the ligated gut loop is not an essential attribute of strains causing septicaemia. Serotypes isolated from calf scours have not been found to affect man or other animals; similarly serotypes related to pig diseases appear to be concerned solely with this animal.

The distribution of serotypes in healthy persons and animals is very different, for human pathogenic types have been isolated from domestic pets. Further work must be done to determine whether such strains can cause disease in man or other animals.

7. SUMMARY

The relationship of certain *E. coli* serotypes to infantile diarrhoea is reviewed. The distribution of these serotypes in healthy individuals and in animals is given. The distribution of *E. coli* causing disease in various domestic animals is reviewed, together with information in respect of host specificity. Experimental investigations using rabbits are described and the interpretation of results in the light of our knowledge of the naturally occurring diseases is reviewed.

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NOTES ADDED IN PROOF

Since this paper was written, Sojka & Carnaghan (1961) have shown that more than 59% of *E. coli* strains isolated from coli septicaemia in fowls belonged to O groups 2, 78 and 1; the remaining 41% were in one of 21 O groups, or unidentified. The common O types were also found in other diseases of poultry. Similar results have been found in the United States by Gross (1958).

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STUDIES ON THE GROWTH, SPORULATION AND CARRIAGE OF *CLOSTRIDIUM WELCHII* WITH SPECIAL REFERENCE TO FOOD POISONING STRAINS

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1. Introduction

Colonies of classical β-haemolytic *Clostridium welchii* type A produce typical zones of complete (β-) haemolysis on horse blood agar. Strains which do not produce complete haemolysis on this medium are variously described as alpha- (α-) or alpha-prime (α′-) haemolytic, weakly haemolytic or nonhaemolytic. The term nonhaemolytic is used collectively here to denote all such strains. Hayward (1943) described nonhaemolytic strains of *Cl. welchii* and Evans (1945) found that these produced α-toxin but no θ-toxin. Attention was directed to nonhaemolytic *Cl. welchii* when Hobbs, Smith, Oakley, Warrack & Cruickshank (1953) showed that strains associated with certain food poisoning outbreaks were typically nonhaemolytic and produced exceptionally heat resistant spores. Typical food poisoning strains, i.e. α-haemolytic or nonhaemolytic on horse blood agar and resistant to 100° for 1 hr in the spore form, were isolated from a wide variety of sources but were not so commonly encountered as classical β-haemolytic strains, which are thought to occur constantly in human faeces.

The occurrence of heat resistant nonhaemolytic *Cl. welchii* in normal human faeces has been reported by various authors; figures for the general population range from 2·2% (Hobbs *et al.* 1953) to 20% in healthy hospital personnel and their families (Dische & Elek, 1957). Hospital patients and personnel seem to have unusually high carrier rates for these strains of *Cl. welchii* and the most recent report from a hospital (Leeming, Pryce & Meynell, 1961) indicates that carrier rates now may be above 30% in some partly closed communities. Carrier rates are high following recognized outbreaks of *Cl. welchii* food poisoning, e.g. 50% after 2 weeks (Hobbs *et al.* 1953) and 25% after 3 weeks (Collee, 1955), and many people exposed may become transient carriers without having felt unwell. Moreover, institutional meals may maintain unduly high populations of *Cl. welchii* (Hobbs *et al.* 1953; McKillop, 1959). Different methods of sampling and treating faeces may account for some of the variations in these figures, but the methods used by Leeming and his colleagues were those currently accepted and involved heating faeces in cooked meat broth for 1 hr at 100°; contamination of the specimen from the bedpan was avoided (Leeming, pers. comm.). It seems that the high carrier rates that prevail in certain institutions may lessen the significance of isolating typical food poisoning strains from patients and carriers during recognized outbreaks of enteritis in these institutions, unless the individual strains can be identified.

In addition to the routine identification of *Cl. welchii*, the differentiation of food poisoning strains at present rests upon their production of typical weakly haemolytic or nonhaemolytic colonies on horse blood agar, the heat resistance of their spores and the agglutination reactions of suspensions with a range of antisera (Hobbs' types). However, the haemolytic effects on blood agar plates are not entirely consistent and may vary slightly with different batches of medium. Spores do not normally occur in significant numbers in cooked food, and spore yields from cultures in the sporulation medium described by Ellner (1956) are variable. Serological identification requires further development, for untypable strains occur and cross reactions are encountered. Furthermore, it is unlikely that all nonhaemolytic strains are potentially heat resistant and it cannot yet be assumed that all heat resistant strains can cause food poisoning. Willis (1957) isolated 50 strains of *Cl. welchii* from a city water supply; these included haemolytic and nonhaemolytic strains. He demonstrated considerable heat resistance of spores from both the haemolytic and nonhaemolytic strains but was unable to be sure that his nonhaemolytic strains were entirely typical of those associated with food poisoning. Willis suggested that perhaps any strain of *Cl. welchii* may be capable of causing food poisoning. Although no volunteer experiments have been done with β-haemolytic strains, it is known that outbreaks occur associated with β-haemolytic strains which are not heat resistant and which gain access to food during the cooling period under conditions suitable for anaerobic growth (McNichol & McKillop, 1958).

The great majority of reported outbreaks of *Cl. welchii* food poisoning are, nevertheless, associated with nonhaemolytic strains and it is pertinent to ask if nonhaemolytic strains possess any advantage, other than their heat resistance, over haemolytic strains with regard to growth at different temperatures. Meanwhile, although the tests usually employed for heat resistance and lack of haemolytic activity are arbitrary, they make use of the only known differentiating features of typical food poisoning strains from the normal haemolytic strains occurring in faeces. While these properties continue to be relied upon for the isolation and preliminary identification of these pathogens, it is important that more should be known about the normal carriage of haemolytic and nonhaemolytic strains in faeces, their growth characteristics, the limits of heat resistance of their spores and the specificity of their agglutination reactions. The work reported here was designed to provide information on some of these points.

2. Materials and Methods

Organisms. Four Colindale reference strains of *Cl. welchii* type A were studied. These were: *Cl. welchii* F 2985/50, a nonhaemolytic, heat resistant strain of Hobbs' serological type 1, isolated in 1950 from cooked salt beef; *Cl. welchii* F 5146/52, a nonhaemolytic heat resistant strain of Hobbs' serological type 10, isolated in 1952 from roast meat; *Cl. welchii* F 6599/59, a classical type A β-haemolytic strain, untypable by Hobbs' sera 1—13, isolated post mortem from a necrotic jejunum in 1959; and *Cl. welchii* F 4637/60, a type A β-haemolytic strain, untypable by sera 1–13, isolated from faeces together with a nonhaemolytic strain during investigation of an outbreak of food poisoning in 1960. All of these strains have been subcultured many times in cooked meat broth; their colonial and serological characteristics appear to be stable. Regular checks for purity of all cultures were made during the experimental work and always at the end of a growth study experiment when the consistency of the serological reactions of the nonhaemolytic strains was verified. Reference strains (also maintained in cooked meat broth) of three other organisms used were: *Escherichia coli* I, E 65/56, from the *Escherichia coli* Reference Laboratory, Colindale; *Bacillus cereus*, NCTC 9945; and *Streptococcus pyogenes*, F 3615/61, a Lancefield group A strain held at the Food Hygiene Laboratory, Colindale.

Culture media. These included horse blood agar, Hedley Wright broth without glucose, nutrient broth with 1% of glucose (glucose broth), cooked meat medium, Willis & Hobbs' (1959) lactose-egg yolk-milk agar with neomycin sulphate (Upjohn) in a concentration of 100 μg/ml, and Ellner's medium (Cruickshank *et al.* 1960). Glucose (1%) was added on occasion as indicated to cooked meat medium (glucose-cooked meat medium). A recovery medium, modified from the holding medium of Wetzler, Marshall & Cardella (1956), contained: minced cooked meat, 5 g; minced cooked egg white, 1 g; chalk, 0·1 g; and soluble starch, 0·1 g; suspended in 20 ml of fluid thioglycollate medium (Oxoid) and sterilized in an autoclave at 121° for 15 min. A sulphite-iron agar medium (Mossel, de Bruin, van Diepen, Vendrig & Zoutewelle, 1956) was used for roll tube counts.

Specimens of faeces. These were submitted from healthy students. All samples were collected in sterile universal containers (Cruickshank *et al.* 1960) with small sterile wooden spoons, avoiding external contamination of the specimen. Suspensions of faeces were made in the laboratory by coating a sterile swab with each sample and emulsifying in tubes of nutrient broth (2 ml). The suspensions were then heated at 70° in a water bath for 10 min (Series 1) or for 1 hr at 100° in a steamer (Series 2), as indicated in the text. After heating, the tubes were cooled for 10 min in a draught of chilled air at 4°. Suspensions of faeces were similarly made, when required, in tubes of cooked meat medium (Series 2).

Sporing cultures of Cl. welchii. Tubes of freshly prepared Ellner medium (8 ml amounts) were inoculated with 0·5–1 ml volumes of 4–6 hr cooked meat broth cultures of *Cl. welchii*. Spore yields varied with different cultures of the same strain in samples of the same batch of medium. Some batches of Ellner medium yielded very few spores; chloroform extraction of the peptone, and preparation of our own yeast extract, did not overcome these inconsistencies. With the exception of the food poisoning strain F 2985/50, which was found to produce spores in cooked meat broth in larger numbers than is usual for *Cl. welchii*, typical food poisoning strains did not generally produce more spores than classical strains in Ellner medium or in cooked meat broth.

Cultures for growth studies. Inocula were prepared by heating 50 ml amounts of Hedley Wright broth in medical flat bottles in a pan of boiling water for 15 min to drive off dissolved air. After cooling, each bottle of broth was inoculated with about 0·2 ml of an overnight cooked meat broth culture of *Cl. welchii* and incubated for 18 hr at 37°. Bottles of unheated broth were used to prepare inocula of the aerobes used. In all cases, 0·02 ml amounts of the 18 hr broth cultures were used to inoculate the bottles of cooked meat broth in which the growth studies were performed. The use of a homogeneous medium would have facilitated the growth studies, but a cooked meat medium was selected to allow incubation of the *Cl. welchii* cultures under aerobic conditions. Medical flat bottles (50 ml) were packed with about 5 g of cooked minced veal or beef and 25 ml of veal or beef broth were added prior to autoclaving at 121° for 15 min. This medium was modified for some experiments by adding sterile 10% glucose solution to give a final concentration of 1% of glucose.

Incubation. The growth of *Cl. welchii* was studied at 23°, 37°, 43°, 47°, 50° and 53°. A water bath with a mechanical stirrer was used and adjusted to the required temperature (variation ±0·5°). The bottles of cooked meat broth were placed in the water bath for at least 30 min before inoculation.

Sampling and bacterial counts. Considerable care had to be exercised in sampling so that the contents were thoroughly mixed without causing aeration of the *Cl. welchii* cultures, which would have upset anaerobiosis. Mixing was achieved by holding each bottle horizontally and rocking 6 times longitudinally and 6 times laterally. The bottles were thereafter held upright until the meat particles had sedimented (15 sec), and then 1 ml aliquots were removed into quarter strength Ringer's solution from which serial tenfold dilutions were prepared immediately. Counts were estimated without delay by the Miles & Misra (1938) technique, using platinum dropping pipettes to deliver drops of 0·02 ml on to dried blood agar plates which were then incubated anaerobically at 37° for 18 hr. Counts were done immediately after inoculation of the bottles of cooked meat broth and after incubation for 2, 4, 5½, 6½ and 24 hr.

In the detailed study of the 'Phoenix phenomenon' (see later), counts were performed at 0, ½, 2, 4, 6, 8, 10 and 24 hr after inoculation, supplementing total counts as above with roll tube counts using sulphite-iron agar. Aliquots (5 ml) of this medium in sterile Astell roll tube bottles were inoculated in parallel with 0·5 ml of undiluted culture and 0·5 ml of a 10^{-1} dilution of it and, after spinning for 2 min to distribute the inoculum, each bottle was incubated anaerobically for 48 hr at 37°. On this medium *Cl. welchii* produces colonies surrounded by a black zone and bacterial counts were thus easily obtained in the range of 2–500/ml. Spore counts were performed by heating 2 ml of the cooked meat medium supernatant to 80° for 5 min and inoculating into sulphite-iron agar in roll tube bottles. The growth resulting from spore inocula in cooked meat broth cultures at 50° was studied using an 18 hr Hedley Wright broth culture inoculated with a spore suspension obtained from a 36 hr Ellner culture and heated at 70° for 15 min to destroy vegetative *Cl. welchii*.

Slide agglutination tests. A range of 13 agglutinating antisera prepared in rabbits at Colindale against typical food poisoning strains of *Cl. welchii* was used at a dilution of 1 in 5 for routine slide agglutination tests with saline suspensions of typical colonies grown in pure culture on blood agar plates.

3. EXPERIMENTAL PROCEDURE AND RESULTS

(a) *The isolation and identification of* Cl. welchii *from faeces*

Series 1. Twenty of 30 specimens (66%) of faeces from healthy students yielded *Cl. welchii* following direct or repeated direct culture of aqueous suspensions of faeces on Willis & Hobbs' medium containing 100 μg of neomycin sulphate/ml. After heating the faecal suspensions at 70° for 10 min, only 8 of the 30 samples (27%) yielded *Cl. welchii* on direct culture on Willis & Hobbs' medium, but *Cl. welchii* was recovered from all of the heated suspensions after they had been subcultured in a fluid recovery medium (see Methods). Most of the *Cl. welchii* strains isolated were β-haemolytic on horse blood agar. Mixtures of haemolytic and nonhaemolytic strains were encountered in 8 instances, and 4 specimens negative on direct plating for *Cl. welchii* yielded only nonhaemolytic strains of *Cl. welchii* from the recovery broths after heating.

Series 2. A second, larger series of faecal specimens from another group of healthy students was investigated by a different method to confirm these findings and to determine the heat resistance of the strains of *Cl. welchii* involved. Thirty-four of 50 specimens (68%) yielded *Cl. welchii* following direct or repeated direct culture of unheated broth suspensions of faeces on blood agar containing 70 μg of neomycin sulphate/ml. Fourteen direct cultures gave profuse or moderate growth and 20 direct cultures gave scanty growth of *Cl. welchii*. From the remaining 16 specimens *Cl. welchii* was isolated only after prior incubation of fresh unheated faecal suspensions in cooked meat broth at 37° with subsequent subculture to neomycin-blood agar plates after incubation for 4 hr and again after 20 hr. Six of the 4 hr subcultures and all of the remaining ten 20 hr subcultures were positive for *Cl. welchii*. Thus, *Cl. welchii* was recovered from all of 50 specimens of faeces. As in Series 1, the majority of the strains isolated produced β-haemolytic colonies on blood agar but careful subculture yielded nonhaemolytic strains from 39 specimens (78%).

Identification. Typical colonies on blood agar were subcultured in cooked meat broth and on Willis & Hobbs' medium. Organisms regarded as *Cl. welchii* were morphologically typical, stout, Gram-positive, asporogenous rods. All grew readily in cooked meat broth and produced gas but no digestion of the meat. All strains fermented lactose on Willis & Hobbs' medium and all produced a lecithinase effect which was specifically inhibited by *Cl. welchii* antiserum.

Heat resistance of faecal strains. After exposure of the faecal suspensions in Series 2 to 100° for 1 hr, a loopful of each was plated directly on blood agar and the remainder cultured in a fluid recovery medium for 4 weeks with regular subcultures onto neomycin-blood agar. Two of the heated suspensions (133, 134) yielded nonhaemolytic *Cl. welchii* on direct culture and a third strain (116) was obtained when the recovery media were first subcultured on neomycin-blood agar after overnight incubation. All of the inoculated recovery media which failed to grow *Cl. welchii* after 4 weeks were heated at 75° for 30 min, cooled and reincubated at 37°, in order to activate any viable ungerminated spores. One additional nonhaemolytic strain (107) was isolated after this treatment. All 4 strains isolated from heated faeces (107, 116, 133 and 134) were subsequently grown in Ellner's medium, and sporing cultures of 3 of them (116, 133 and 134) were shown to survive exposure to 100° for 1 hr in cooked meat broth. However, there were some unsuccessful attempts to produce sporing cultures before this was achieved, and modified heat resistance tests (100° for 30 min) were consistently negative with Ellner cultures of strain 107 which produced few spores. The possibility that strain 107 was introduced as a contaminant during repeated subculture of the recovery medium must therefore be considered. Heat resistance tests using 2 of the original faeces samples which had been stored at 4° for 8 weeks confirmed the presence of heat resistant *Cl. welchii* (specimens 116 and 133). Specimen 134 was too scanty for further testing and specimen 107 continued to give negative results. Thus 3 or 4 (6–8%) of 50 normal faeces samples yielded heat resistant *Cl. welchii* resembling food poisoning strains.

The heat resistance of the remaining nonhaemolytic strains was then investigated. Microscopic examination of stained smears from 36 hr Ellner cultures of all of the nonhaemolytic strains isolated in Series 2 showed large numbers of spores (several or many/microscopic field under a $\frac{1}{12}$ in. oil immersion objective) in 16 of 39 cultures. These included one of the known heat resistant strains (133). When 1 ml volumes of 12 of these profusely sporing cultures were heated for 1 hr at 100° in cooked meat broth, only the known heat resistant strain (133) included in this test survived. Modified heat resistance tests (100° for 30 min in cooked meat broth) of 1 ml suspensions of Ellner cultures of all 39 nonhaemolytic strains and 3 haemolytic strains, and repeat heat resistance tests of all of the faeces stored at 4° for 2–3 months, revealed no additional heat resistant strains.

Toxigenicity. Culture filtrates of 3 representative nonhaemolytic heat sensitive strains were submitted to Dr. G. Harriet Warrack, who reported that all contained α- and κ-toxins but lacked β-, δ-, κ-, ε-, ι-, and λ-toxins. These appeared, therefore, to be type A strains with a toxigenicity similar to that of food poisoning strains of *Cl. welchii*.

Serology. Fifteen of the 39 nonhaemolytic strains examined gave positive slide agglutinations when tested against a range of 13 antisera prepared against food poisoning strains, but the results of preliminary absorption tests indicate that these may include some nonspecific reactions.

(b) *Comparison of the heat resistance of spores of β-haemolytic and food poisoning strains*

Heat resistance was more apparent with suspensions containing large numbers of spores. These were grown in Ellner medium for 3–4 days at 37° and thereafter held in the culture medium on the bench for several weeks before testing. Inocula of $1{\cdot}4\times10^4$ spores of the β-haemolytic strain F 4637/60 in tubes of cooked meat broth survived exposure to 100° for 10 min but not 20 min. A tenfold increase in the spore inoculum (to $1{\cdot}3\times10^5$) did not result in survival for 20 min at 100°. Suspensions of $1{\cdot}6\times10^6$ spores of the food poisoning strain F 2985/50 yielded survivors after 8 hr at 100° in cooked meat broth (Table 1).

Suspensions of smaller numbers of young spores grown for 3–4 days in Ellner medium, washed and suspended in distilled water, survived heating for shorter periods. When 1 ml amounts of such inocula containing about 1,500 spores of the β-haemolytic strain F 4637/60 were held in tubes of cooked meat broth for varying times in the steamer, then cooled and incubated anaerobically at 37°, growth did not occur in tubes which had been held for more than 5–10 min at 100°. Similar suspensions of the food poisoning strain F 1546/52 consistently survived exposure to 100° for 4 hr but not 5 hr under these conditions (Table 1). When similar amounts of the same spore suspension were sealed in tubes of cooked meat broth and the large ampoules totally immersed in boiling water, growth also occurred in the incubated tubes which had been exposed to 100° for up to 4 hr.

The same concentrations of washed spores of the food poisoning strain F 1546/52 in 1 ml amounts of distilled water at pH 6·5, sealed in small thin-glass ampoules, immersed in boiling water at 100° and subsequently inoculated into cooked meat broth showed survival after 20 min but not after 40 min (Table 1). This marked reduction in survival time with ampoule tests also occurred when the spores were suspended in 0·02 M sodium phosphate buffer at pH 7·0 or in the sterile supernatant of cooked meat broth. In general, attempts to improve recovery from the heated ampoules by using fresh presteamed cooked meat broth or complex fluid recovery media at 37° under anaerobic conditions were no more successful, but one buffered ampoule test at 100° for 3 hr yielded survivors on recovery in cooked meat broth enriched, prior to autoclaving, with horse blood (1·5 ml) and soluble starch (0·1 g). Ampoules containing 1,500 spores of the food poisoning strain F 1546/52 in 1 ml of distilled water yielded survivors after 2 min but not after 4 min at 110° in an oil bath. Thus, ampoule tests did not differentiate so clearly between the spores of the β-haemolytic and the food poisoning strains as the tests using tubes of cooked meat broth at 100°, when a protective effect occurred which was more apparent with the heat resistant strains (Table 1).

The effect of cooked meat broth on observed heat resistance was confirmed by comparing the resistances of graded inocula of old spores from the heat resistant strain F 2985/50 (see Methods) when held in distilled water in test tubes or in tubes of cooked meat broth and exposed to steam at 100° for varying periods (Table 2). Small numbers of spores heated in cooked meat broth survived steaming for 4 hr (the maximum exposure time used in this series of tests), whereas an identical inoculum of spores suspended in 1 ml of distilled water in a test tube did not survive the minimum exposure of 50 min (Table 2). These findings may be important from the practical aspect of bulk cooking of meat in relation to *Cl. welchii* food poisoning.

Table 1. *The results of heat resistance tests performed at* 100° *in cooked meat broth medium or in ampoules on classical haemolytic* (*H*) *and food poisoning nonhaemolytic* (*NH*) *strains of* Cl. welchii

Strain no.	Nature of strain	Spore inoculum	Maximum survival time (min)
F 4637/60	H	$1{\cdot}4 \times 10^4$	10
		$1{\cdot}3 \times 10^5$	<20
F 2985/50	NH	$1{\cdot}6 \times 10^6$	>480
F 4637/60	H	$1{\cdot}5 \times 10^3$	<10
F 1546/52	NH	$1{\cdot}5 \times 10^3$	240
F 1546/52	NH	$1{\cdot}5 \times 10^3$*	20

* Ampoule test.

Table 2. *The results of heat resistance tests performed at* 100° *on the nonhaemolytic strain F* 2985/50 *in distilled water* (*DW*) *and in cooked meat broth* (*CMB*)

Duration of heating (min)	Spore inoculum	Suspending medium	Survival*
50	$1{\cdot}5 \times 10^3$	DW	−
60	$1{\cdot}5 \times 10^3$	DW	−
60	$1{\cdot}5 \times 10^3$	CMB	+
120	$1{\cdot}5 \times 10^5$	DW	−
120	$1{\cdot}5 \times 10^3$	CMB	+
180	$1{\cdot}5 \times 10^6$	DW	−
180	$1{\cdot}5 \times 10^3$	CMB	+
240	$1{\cdot}5 \times 10^3$	CMB	+

* Survivors: present, +; absent, −.

(c) *Comparison of the growth patterns of β-haemolytic and food poisoning strains at different temperatures*

Viable counts were made of 2 typical food poisoning strains (F 2985/50 and F 1546/52) and 2 β-haemolytic strains (F 6599/59 and F 4637/60) in a series of cooked meat broth cultures at intervals during the first 24 hr of growth at 23°, 37°, 43°, 47°, 50° and 53° (Fig. 1). No significant differences were noted between the growth patterns of the haemolytic and nonhaemolytic strains at 23°, 37°, 43° and 53°. At 23° all strains showed a small initial reduction in numbers up to 5 hr followed by a rise, generally to high counts at 24 hr. Cultures at 37° showed no lag phase and multiplied rapidly to maximum counts at approximately 5 hr; subsequently there was little reduction in counts up to 24 hr. At 43° this pattern was exaggerated, with slightly higher maximum counts at about 5 hr followed by gradual declines to lower values at 24 hr. At 53°, the counts of all strains fell rapidly to below 500/ml at $5\frac{1}{2}$ hr and did not rise during the 24 hr test period.

At 47°, three of the strains had similar growth patterns, with an initial rapid increase to maximum counts at 5 hr and a gradual decline thereafter, but the

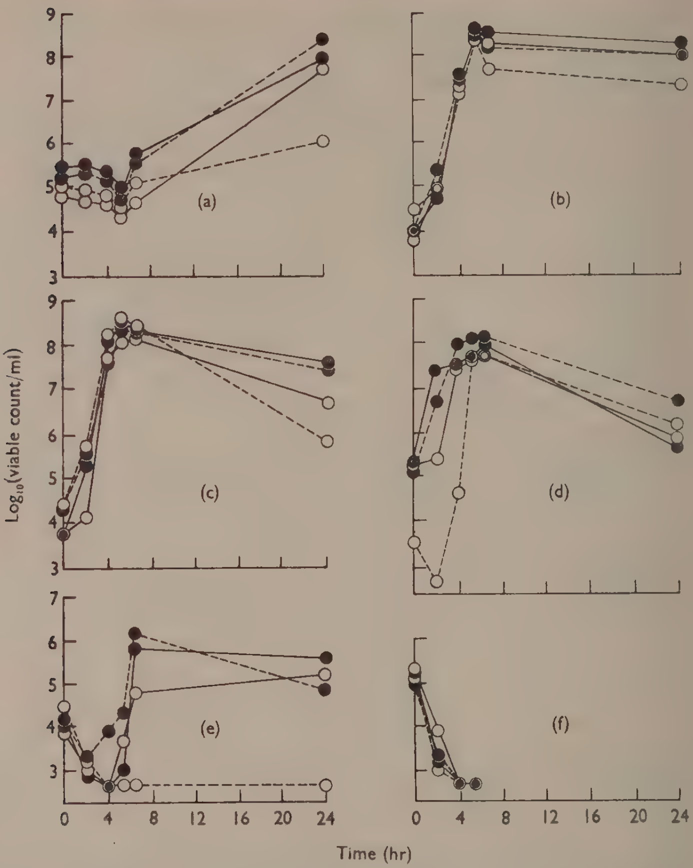

Fig. 1. The growth of 4 strains of *Cl. welchii* type A at different temperatures: (a), 23°; (b), 37°; (c), 43°; (d), 47°; (e), 50°; (f), 53°. Broken lines, heat sensitive haemolytic strains F 4637/60 (open circles) and F 6599/59 (full circles); unbroken lines, heat resistant food poisoning strains F 2985/50 (open circles) and F 1546/52 (full circles).

β-haemolytic strain F 4637/60 showed a marked fall in numbers initially, the survivors then multiplying rapidly to a maximum count at 6 hr with a gradual drop in numbers thereafter. This unusual growth pattern was produced by the other three strains when they were grown at 50°, with initial falls to minimal counts at 4 hr followed by sudden increased growth to maximum counts at 6 hr. For convenience, this is referred to as the 'Phoenix phenomenon'.

Strain F 4637/60 behaved in this manner at 47° but not at 50°, when there was a rapid and sustained reduction in count to below 500/ml. Further growth studies of additional strains, however, confirmed that 7 out of 7 nonhaemolytic and 4 out of 6 β-haemolytic strains readily reproduced the Phoenix phenomenon at 50°. There was thus no significant difference between nonhaemolytic and haemolytic strains in this respect but, as the observation may be important in matters of food hygiene, the mechanism was further investigated.

Studies with heated suspension of strain F 2985/50, calculated to contain 250,000 spores/ml and no viable vegetative organisms (see Methods), showed that the Phoenix type of growth curve could be obtained with spores at 50°.

In order to exclude spores from the initial inocula, serial subcultures from glucose broth were used to inoculate tubes of cooked meat broth containing 1% of glucose at 50°. Under these conditions, counts of the β-haemolytic strains F 6599/50 and F 4637/60, and also the nonhaemolytic strain F 1546/52, fell rapidly to less than 500/ml within 2 hr and the cultures did not yield viable *Cl. welchii* on subculture after 24 hr. But the counts of the nonhaemolytic strain F 2985/50 declined more gradually to less than 500/ml in 6 hr, and subsequently rose again to $5{\cdot}75 \times 10^6$/ml at 24 hr. It seemed significant that in this experiment spores (at a concentration of 6/ml) could be detected in the inoculum for this strain only. A more detailed study of the growth of F 2985/50 in glucose-cooked meat broth at 50° demonstrated a gradual fall in viable count from 25,000/ml to 2/ml in 8 hr, rising to 6,000/ml at 24 hr, i.e. a rather extended Phoenix effect. The spore content of the initial inoculum and of the culture throughout this experiment was less than 2/ml; it is possible that the initial population was reduced to an undetectable spore level at 8 hr. Further attempts to elucidate the phenomenon using combinations of inoculum and culture medium with and without added glucose were frustrated by the tendency of this strain to produce unusually large numbers of spores, e.g. 202/ml even in some glucose broth cultures and 1,160/ml in broth cultures without glucose. The Phoenix phenomenon was reproduced in all of these growth studies and the possible participation of spores could not be excluded.

Attempts were made to replicate the Phoenix growth pattern by using *Cl. welchii* grown at 50° to inoculate fresh medium at 50°. Two methods were used. First, cooked meat broth at 50° was inoculated with 1 ml of a 24 hr 50° culture of F 2985/50, and second, a 24 hr cooked meat broth culture at 50° was subcultured on blood agar at 37° for 24 hr. Single colonies were then picked into bottles of preheated broth at 37° and incubated for 18 hr, at which time 0·02 ml of each of the cultures was transferred to bottles of cooked meat broth at 50°. In both of these experiments the Phoenix effect was reproduced. This suggests that the phenomenon is not solely a matter of selection of cells adapted to the higher temperature. Comparison of films from cooked meat broth cultures of F 2985/50 at 37° and 50° stained by Gram's method showed that growth at the higher temperature was associated with the production, together with organisms of normal dimensions, of many abnormal forms including long chains and filaments.

The growth patterns at 50° of one strain each of *Strep. pyogenes*, *B. cereus* and *E. coli* in cooked meat broth cultures were studied. The viable count of *Strep. pyogenes* fell within 30 min from the initial 575,000/ml to less than 500/ml and no organisms could be recovered after 24 hr at 50°, despite subsequent subculture for 24 hr at 37°. The initial count of *B. cereus* (40,000/ml) fell to less than 500/ml after 2 hr at 50° and remained thus up to 24 hr, when subculture at 37° proved that viable organisms were nevertheless present. The initial *E. coli* count of 275,000/ml fell slowly to a minimum of 5,000/ml at approximately 4 hr. There followed a gradual increase to 170,000/ml at 24 hr. Thus, a growth curve similar to that of an extended *Cl. welchii* Phoenix form was obtained with a nonsporing organism: but a definite Phoenix effect was obtained with none of these 3 organisms.

4. Discussion

Haemolytic strains of *Clostridium welchii* occur widely in nature and are said to be present 'constantly' and 'uniformly' in human faeces (Wilson & Miles, 1955; Smith, 1955). Bacteriophages and other organisms are active against *Cl. welchii* (Smith, 1955; Smith, 1959; Stark, 1960) and some strains of *Cl. welchii* produce diffusible products which inhibit the growth of other strains (Smith, 1959). In addition germination of spores may be irregular. It is therefore not surprising that *Cl. welchii* is not readily isolated from all faeces.

Investigations on the carriage of *Cl. welchii* and studies of its spores have been facilitated by the development of selective solid media (Lowbury & Lilly, 1955; Willis & Hobbs, 1959) and a sporulation medium (Ellner, 1956). Lowbury & Lilly (1955) isolated *Cl. welchii* from 70 out of 112 (62%) specimens of faeces by direct plating on neomycin-Nagler plates, and this figure was later improved to 89 out of 112 (79%) by prior enrichment in cooked meat broth (Lilly, 1958).

Using similar methods, we have now demonstrated the constant occurrence of *Cl. welchii* in human faeces. Our figures for *direct* isolations agree with those of other workers and indicate that about two-thirds of the specimens examined contained *Cl. welchii* in relatively large numbers.

The carriage of heat resistant *Cl. welchii* by 6–8% of a student population participating in regular communal feeding was not unexpected, as bulk cooking favours transmission of these strains. The high incidence of nonhaemolytic strains in the faeces investigated is of interest but, as those strains did not survive the routine heat resistance tests performed on faeces or sporing cultures, they should not be confused with typical food poisoning strains. Our findings suggest that the heat resisting property of spores of heat resistant strains of *Cl. welchii* is not merely dependent upon the production by these strains of large numbers of spores. Vas & Proszt (1957) showed that exceptionally heat resistant spores of *B. cereus* occurred in a fraction of a population of spores which had a natural distribution of resistance; they did not arise by mutation and their sporing descendants did not show increased heat tolerance. Our results with *Cl. welchii* confirm earlier impressions that typical food poisoning strains produce spores which consistently exhibit a degree of heat resistance much greater than that of typical classical strains. Moreover, we found that this is so even in small populations of spores and that these strains can produce highly resistant progeny *in vitro*. Thus, although the thermostability of heat resistant *Cl. welchii* may be influenced by environmental conditions conducive or inhibitory to abundant sporulation, and accepting that the test conditions will influence the degree of heat resistance reported, it seems that the heat resistant property of the formed spore of heat resistant strains of *Cl. welchii* is genetically determined.

Selection and transmission of heat resistant strains by bulk cooking of meat under conditions somewhat similar to those which favour survival of heat resistant spores *in vitro* would partly account for their predominance in institutions. The presence of reducing substances in the heated meat medium may minimize the lethal effect of heat on *Cl. welchii* spores and improve conditions for their subsequent growth. Several factors may be involved and, as any such protective effect of cooked meat is of importance in food hygiene, the observation merits further investigation. The current laboratory practice of isolating heat resistant *Cl. welchii* from faeces by steaming a suspension of the specimen emulsified in cooked meat broth for 1 hr involves similar protection by organic matter, and it seems that heat resistance tests on aqueous suspensions of spores would be less successful in differentiating heat resistant and heat sensitive spores. A more quantitative index of carriage of heat resistant strains could be obtained by direct plating of heated faecal suspensions on neomycin-blood agar plates. The two direct positive specimens from carriers of heat resistant strains in our investigation yielded very few nonhaemolytic colonies (9 and 5/loopful (0·02 ml) of heated faecal suspension plated), and it would now be of interest to compare figures for direct isolations after heating faecal specimens from food poisoning cases and those who had eaten suspect food.

The results of our limited serological investigation confirm the observations of Hobbs *et al.* (1953) that antigens may be shared by heat resistant and other strains of *Cl. welchii* and suggest that some nonspecific agglutination reactions may occur. Results of slide agglutination tests with the range of 13 antisera prepared against known food poisoning types are of proven value from the epidemiological aspect, however. Of 298 typical food poisoning strains of *Cl. welchii* associated with outbreaks of food poisoning and submitted to Colindale for serological typing during the period August 1960–March 1961, 222 (74·5%) were typable. During the same period, 49 cultures of β-haemolytic strains were received and only 2 (4·1%) agglutinated with one of the specific antisera.

The unusual pattern of growth at 50°, the 'Phoenix phenomenon', was exhibited by most of the *Cl. welchii* strains examined. Jensen (1944) regarded food held for 4–8 hr in the range 120–50° F (48·9–10° C) as potentially dangerous owing to the bacterial multiplication which may occur, depending upon the type of food and species of organism concerned. It is now evident that growth of *Cl. welchii* may occur in cooked meat just beyond the upper limit of this range and that growth can be initiated by inocula of spores protected against heat inactivation in this medium. The survival of spores in meat is further ensured by the fact that many cooking procedures do not produce adequate heat penetration (see Norval & Collee, 1957; Sylvester & Green, 1961). Although the protective effect of heating in cooked meat broth is most apparent with spores of heat resistant strains, we have found that the Phoenix phenomenon is not restricted to these strains. Thus, heat sensitive *Cl. welchii*, when protected by lack of heat penetration or introduced into a favourable anaerobic environment during the cooling period, will grow just as rapidly as heat resistant strains over the temperature range of our tests.

The mechanism of the Phoenix effect at 50° is obscure. Some of our evidence suggests that spores may play a part; they may be required to ensure survival of the initial heat shock when the inoculum is transferred to broth at 50°. The prompt decrease in viable count may be ascribed to this effect, with subsequent germination and selection of heat adapted variants. Although growth then proceeds rapidly for a period, normal maximum counts are not attained and some reduction in viable counts at 50° may be associated with disfunction of cell division systems. This view is supported by our observation of filamentous forms and chains of organisms produced at this temperature. Our failure to produce the Phoenix phenomenon with a sporing aerobe is not inconsistent with the above theory, as different growth characteristics, e.g. inability to grow profusely at low oxygen concentrations, are involved with this species. On the other hand, ability to repeat the Phoenix effect from one 50° culture of *Cl. welchii* to another at 50° does indicate a lag phase in the second culture, which might have been less pronounced if heat adaptation were the sole explanation. Attempts to replicate the effect serially from younger and older cultures at 50° may yield more information on this problem. Williams (1956) attributed an unusual series of growth phases of a thermoduric micrococcus at 37·5° solely to the influence of the temperature of incubation. He postulated the outgrowth of mutants or variants adapted to grow at that particular temperature. The remote possibility that unstable mutants are produced at 50° must also be considered, as Zamenhoff (1958) has demonstrated the mutagenic effect of heat at 55–60° on *Escherichia coli*. We observed no significant colonial or serological changes in strains of *Cl. welchii* grown at 50° and subcultured at 37° on blood agar, and no changes in haemolytic activity were evident. Zamenhoff (1960) also observed that cells of *E. coli* which survived heat shock under certain circumstances sustained non-hereditary damage resulting in delayed growth. It may be that our observations on the growth of *E. coli* at 50° can be partly explained on this basis. The Phoenix effect produced by *Cl. welchii* is associated with quite a different pattern of growth at 50°, however, and final elucidation of this phenomenon awaits further investigation.

5. SUMMARY

Procedures are described by which the constant occurrence of *Clostridium welchii* in human faeces has been demonstrated. Nonhaemolytic strains of *Cl. welchii* occurred in 39 of 50 specimens of faeces examined, but spores of only 3 of these strains were exceptionally heat resistant. Heat resistance tests with spores produced from cultures of stock strains revealed a protective effect of cooked meat which was most apparent with heat resistant strains. Preliminary findings indicate that the heat resisting property of spores of typical food poisoning strains of *Cl. welchii* is genetically determined and is not primarily dependent upon the presence of large numbers of spores. An unusual growth pattern at 50°, the 'Phoenix phenomenon', was observed with both heat sensitive and heat resistant strains.

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PUBLIC HEALTH SIGNIFICANCE OF SALMONELLA CARRIERS IN LIVESTOCK AND BIRDS

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1. Introduction

Food hygiene and food poisoning investigations include not only a study of the spread of intestinal pathogens but also continual observance of the natural habitats or sources of these organisms. It is unsatisfactory to check the spread of infection without knowing the origin of the pathogens, although much can be done to lessen the risks of outbreaks by careful hygiene in the kitchen, factory or abattoir, and this, in some instances, may be the only means of reducing the incidence of food poisoning.

Ten years ago food hygiene studies centred round the human carrier and efforts were concentrated on hand washing, the control of coughing and sneezing, improvements in washing-up and the elimination of cracked cups. All these play a part, and it may be assumed that careful regard to personal hygiene has prevented many outbreaks of staphylococcal food poisoning.

An understanding of cross contamination and growth rates will help to prevent salmonellae present in certain foodstuffs (raw products or uncooked ingredients of mixed foods) surviving in the finished article, or causing recontamination by intermediate vehicles such as food, equipment, utensils, hands and cloths.

Investigations have shown that a variable proportion of imported and home produced frozen and dried egg products and meats, desiccated coconut, and other substances may contain small, or sometimes large, numbers of salmonellae. The introduction of these foods into the kitchens of hospitals, schools, factory canteens and homes and also into shops can initiate the spread of salmonellae, including *Salmonella paratyphi* B, more or less freely in the environment according to the habits of the staff and the equipment available. These facts have stimulated investigations on the carrier rate of salmonellae in the animals and birds which supply these food products or which may be in contact with them, the foodstuffs which these creatures eat and the ecology of the pathogens on farms, and in markets, slaughterhouses, shops and kitchens.

The most satisfactory solution to the steadily increasing salmonella problem is to eliminate the source of infection in the country of origin, or to destroy the salmonellae in the food before distribution.

Serological and phage typing techniques have enabled the interchange of salmonellae between man, animals and foodstuffs to be followed more closely. The Salmonella Reference Laboratory gives serotype names to many hundreds of salmonellae (Taylor, 1960), and the Enteric Reference Laboratory is using bacteriophage for typing not only typhoid and paratyphoid organisms but also *Salm. typhi-murium* (Callow, 1959) and other serotypes (Anderson, 1960*a*); these detailed analyses of strains are proving to be invaluable.

2. THE SIGNIFICANCE OF POULTRY AND EGG PRODUCTS

Fowls are said to be the greatest reservoir of salmonellae both in the United Kingdom (Buxton, 1957, 1958; Gordon, 1959; Garside, Gordon & Tucker, 1960) and in the United States (Edwards, Bruner & Moran, 1948); these authors also claim that a high incidence of a particular serotype in a given locality is accompanied by a high incidence of the same type in man in that area.

Dixon & Pooley (1961) studied the incidence of salmonellae in a poultry processing plant where there is likely to be far more spread of contamination than in the retail sale in butchers' shops of poultry killed on the farm. The results obtained on 13 of 23 weekly visits to a factory processing 12,000 twelve-week old broiler chicks daily showed that 13·8% of 544 specimens were positive for salmonellae. Positive samples included 21 out of 87 (24·1%) samples of chilled water, 19 out of 60 swabs (31·7%) immersed in chill tanks, 33 out of 334 (9·9%) swabs from eviscerated carcases and edible viscera, and 2 out of 22 (9·1%) swabs from staff members (Table 1). *Salm. typhi-murium* and *Salm. thompson* were the commonest serotypes found.

Table 1. *The incidence of salmonellae in samples from a plant processing* 12,000 *chickens daily*

(From Dixon & Pooley, 1961)

Description of samples	No. examined	Samples containing salmonellae	
		No.	%
Water from chilling tanks	87	21	24·1
Water from scalding and other tanks	41	0	0
Swabs in chill tanks	60	19	31·7
Swabs from carcases and viscera	334	33	9·9
Swabs from staff	22	2	9·1
Total*	544	75	13·8

* Results obtained on 13 out of 23 visits.

It was pointed out that opportunity for dissemination occurred during evisceration and chilling and the authors suggested that the only effective way to exclude salmonellae from broiler processing plants would be to eliminate them from the feedingstuffs of young chickens.

Sadler *et al.* (1961) described investigations in a factory and showed that infected or carrier birds were being processed on 43% of sampling days for turkeys, on 26% for chicken fryers and on 12% for hens. Turkeys infected with salmonellae were detected on twice as many sampling days with mass sampling as with the small sampling technique, and it was suggested that positive sampling periods approaching 100% may have resulted if more samples could have been examined.

It appears, therefore, that poultry carcases contaminated with salmonellae will be received into many kitchens. Where refrigeration is available the numbers of salmonellae in the birds will remain low, but without refrigeration the salmonellae will increase and there will thus be a greater danger of cross contamination from benches and equipment to other products in the kitchen.

There is little information available on the mode of infection of the chickens themselves, whether it be by feedingstuffs only or also by contact with other infected animals or birds. In one epidemiological investigation, *Salm. infantis* was isolated from poultry feedingstuffs, eggs and patients who had eaten lightly cooked and uncooked egg products (Newell *et al.* 1959).

Garside, Gordon & Tucker (1960) described experiments in which day-old chicks were fed *Salm. typhi-murium* in diets containing chlortetracycline. The mortality rate in the chicks fed with antibiotics was approximately half that of chicks not receiving antibiotics, but more carriers occurred in the groups fed with antibiotics. There was a gradual development of resistance to chlortetracycline in sensitive strains fed to the chicks. Hobbs *et al.* (1960) used such infected birds to show that *Salm. typhi-murium* could be isolated from the skin of 10–12 week old birds killed and processed without regard to hygiene, and also from cavity swabs, offal, trays and slush ice used for chilling. ‘Acronization’, the immersion of the dressed poultry in slush ice containing chlortetracycline at 10 p/m, suppressed the growth of sensitive strains of *Salm. typhi-murium* together with spoilage organisms, whereas a resistant strain of *Salm. typhi-murium* grew readily and more rapidly than the spoilage organisms.

The food poisoning hazards of egg products are well known and Table 2 shows the extent of the problem with regard to the proportion of contaminated samples (Report, 1958*a*). Despite this, untreated liquid and dried whole egg, yolk and white are still being used on a large scale in the confectionery and bakery trades and also in catering establishments. Many tons of egg products are imported annually into the United Kingdom. Table 3 gives figures for the contamination of egg products sampled by the City of London. From 1955 to 1958 approximately 382 tons of dried whole and white of egg and 3,000 tons of frozen liquid egg were received by the Port of Liverpool (Parry, 1961), and of these 13·4% of the dried egg and 26·6% of the liquid frozen egg samples were found to be contaminated with organisms of the salmonella group. Parry described the survival of, or recontamination by, salmonellae from Chinese frozen egg albumen in baked confectionery such as lemon meringue pies,

Table 2. *The incidence of salmonellae in egg products from various countries in the period* 1955–1956

(From Report, 1958*a*)

	Type of product	No. examined	Samples containing salmonellae	
			No.	%
Egg	Dried, whole	1,601	122	8
	Frozen, whole	8,962	3,020	34
	Liquid, whole	21	0	0
Total		10,584	3,142	30
Albumen	Flaked	5,581	1,066	19
	Powdered	449	66	15
	Frozen	2,681	315	12
Total		8,711	1,447	17

meringues, macaroons and Japan biscuits, occurring when the hygiene of preparation was careless and the oven temperature irregular. In baking tests carried out with greater care over hygiene and cooking times there was no evidence of salmonellae in the finished products. Cross contamination was considered to be the most serious danger when egg mixes containing salmonellae were used in bakeries, because of the contact of the baked foods or uncooked fillings with the bakers' hands or equipment which had previously been contaminated by raw egg. It was also pointed out that in warm bakeries, especially in the summer months, flies constitute a further hazard, even when the bakery staff are careful to reduce splash and soiling to a minimum; five flies infected with salmonellae were caught in the bakery during the experimental cooking described by Parry. Outbreaks of enteric fever from *Salm. paratyphi* B in Chinese frozen whole egg have been described by Newell, Hobbs & Wallace (1955); samples of egg from other countries have proved to be negative for paratyphoid organisms.

Table 3. *The incidence of salmonellae in egg products imported into the City of London*

Year	No. of containers*	Samples taken		Samples containing salmonellae	
		No.	%	No.	%
1956	26,822	444	1·6	34	7·7
1957	456,994	5,996	1·3	568	9·5
1958	26,304	755	2·9	100	13·2
1959	2,324	296	12·7	57	19·3
Total	512,444	7,491	1·5	759	10·0

* Drums, cartons and packages.

Harvey & Phillips (1961) found that 31 out of 111 (27·9%) swabs taken from bakehouse floor drains were positive for salmonellae, including *Salm. typhi-murium*, *Salm. aberdeen*, *Salm. thompson* and *Salm. paratyphi* B, and all except *Salm. thompson* were isolated from 15 out of 93 swabs from staff sewage. Salmonellae were also isolated from outflow water from staff wash-hand basins and from swabs wiped over machinery, even after routine cleaning. Chinese frozen egg was in daily use and was visible on the hands and forearms of bakery staff and on floors and tables.

Harvey *et al.* (1961) described an outbreak of food poisoning due to a mixed infection of *Salm. typhi-murium* phage type 2c, commonly found in poultry and egg products, and *Salm. thompson* phage type 4. More than 100 children were taken ill after eating trifle which included cake made with an egg product. The two serotypes of salmonellae were found in the trifle, in stools from the bakery staff and in drain swabs.

Broken-out egg products prepared in the United Kingdom are also contaminated, mostly with *Salm. typhi-murium.* In the annual survey of food poisoning incidents published in the Monthly Bulletin of the Public Health Laboratory Service for 1959 (Report, 1960) it is pointed out that the rise in cases of salmonellosis during the last few years has been due to serotypes known to be present in egg and in meat products.

Salmonellae can be killed in liquid whole egg by HTST pasteurization and it is recommended that a temperature of 147–149° F for 2½ min should be used (Murdock *et al.* 1960); albumen can be treated at a slightly lower temperature. Flaked egg albumen prepared by the pan technique can be heat treated at 130° F for 9–10 days to remove salmonellae (Report, 1957).

Final experiments with artificially inoculated liquid egg have been carried out in a commercial plant and the pasteurized product tested for baking quality (Heller, Roberts, Smith & Hobbs, 1962); a test to establish the efficiency of heat treatment (Brooks, 1962) has been correlated with destruction of salmonellae in laboratory scale pasteurization experiments (Shrimpton, Monsey, Hobbs & Smith, 1962).

Additional bird carriers may be found among the millions of pigeons and starlings living in many towns. Excreta contaminated with salmonellae will be dropped in the streets and also on the wharves of docks where surfaces, over which sacks of food are dragged, may be slimy with bird faeces.

3. THE SIGNIFICANCE OF MEAT PRODUCTS AND DOMESTIC ANIMALS

The carriage of salmonellae by cattle and other domestic animals has frequently been described in the literature, but for some years *Salm. dublin* was regarded as the only significant serotype in cattle. This type is not one of the more common causes of salmonellosis in man, and therefore little attention was paid to the public health significance of salmonellae in cattle. Recently, combined veterinary and public health investigations have shown that *Salm. typhi-murium* is far commoner among farm animals, both as carriers and as victims of salmonellosis, than was hitherto known.

Anderson, Galbraith & Taylor (1961) described an outbreak of *Salm. typhi-murium* phage type 20a when veal was the food vehicle. The relevant carcases had come from an area in which farms had suffered outbreaks of salmonellosis among calves due to *Salm. typhi-murium* of the same phage type. Investigations in Bradford (McDonagh & Smith, 1958) gave results that illustrated the increase in salmonellosis among people in the vicinity of an abattoir in which a coincidental rise in salmonella isolations from pigs was demonstrated at the same time. Similar observations are being made in other areas where swabs from sewers in slaughterhouses, bakeries, and shops are used to indicate the occurrence of salmonellae in the vicinity.

Harvey & Phillips (1961) found that 111 out of 274 (40·5%) swabs from gullies in abattoirs were positive for salmonellae; 21 different serotypes were isolated, of which *Salm. typhi-murium* was the commonest and *Salm. dublin* the next in frequency. Of 30 phage typed *Salm. typhi-murium* from human infections (mostly sporadic) in the area, 23 belonged to phage types found in the abattoirs and on local farms at approximately the same time. There was a similar correspondence between human cases of salmonellosis due to other serotypes and the isolation of these serotypes from abattoir swabs.

Hobbs & Wilson (1959) reported that 12–18% of 332 samples of boneless veal imported frozen were contaminated with salmonellae, the predominant serotype being *Salm. typhi-murium*. Of 890 samples of boneless veal, beef and mutton, 10·3% were contaminated. This meat is of poor quality, the veal from newly born calves, and is used mostly for manufacturing purposes as an ingredient of pies and sausages, but it is also used in canteens as a cheap source of protein. The beef may be minced and sold in chilled and frozen packs as well as in sausages. For comparison carcase meat including veal, beef, lamb, mutton and pork was also examined and, of 304 samples, 4·3% were contaminated; the difficulty of sampling carcase meat is well known.

Table 4. *A summary of salmonellae isolations from raw meats during the period July* 1959–*July* 1961

Type of meat	Origin*	No. of samples examined	Samples containing salmonellae	
			No.	%
Boneless veal	A	138	4	2·9
	B	35	1	2·9
Boneless beef	A	5	0	0·0
	B	316	28	8·9
	C	65	2	3·1
	F	689	45	6·5
Boneless mutton	A	141	5	3·5
	C	15	5	33·3
Total		1,404	90	6·4
Carcase beef	D	95	4	4·2
	G	9	0	0·0
Total		104	4	3·8

* Each letter represents a different country of origin.

Table 4 gives the results of examinations of meat samples from 1959 to 1961. It shows a marked improvement in the results for boneless veal, but not for boneless beef or mutton. These results and the little information available on the incidence of salmonellosis among animals suggest that infection may be widespread and that occasionally it reaches a high level as shown by figures of 50% for the carrier rate among small groups of calves in certain areas.

Recent investigations in this country have shown that marketed calves sometimes introduce salmonellae into farming stock and that stable companions can become symptomless excreters and cases. When outbreaks on farms are investigated thoroughly, dogs and humans can be shown to be infected, and it is suggested that human beings may acquire infection from sick calves or carriers and adult cattle. Riddel, Norval & Anderson (1959) reported a milkborne outbreak of more than 100 cases of *Salm. typhi-murium* in Scotland; 60 out of 110 cows on the relevant farm were found to be excreting *Salm. typhi-murium* at some time, and 2 cows and 4 calves died from the infection. There had been uninvestigated cases of gastro-enteritis among 4 households from which 7 of the farm dairy workers came and in 3 other households on the farm who drank the same milk.

The farm troughs and drinking bowls were contaminated with dung, and the cows ate bedding, straw and grain contaminated with rodent excreta from neighbouring stacks infected with mice. It is suggested that infection in a carrier animal may have become acute and spread to other animals or that the contaminated grain and bedding may have been responsible. It is emphasized that stress factors (e.g. calving) can precipitate the onset of salmonellosis. Carriage of salmonellae by cattle is not prolonged and excreta examined a few weeks after the occurrence of outbreaks have been negative for salmonellae. It seems, however, that there is an increase in the number of carriers after animals have been held in markets and abattoirs; similar observations have been made on the build up of infection in markets for pigs in the United States of America (Galton *et al.* 1954).

The infection from the lairage of markets and abattoirs will become more widespread during slaughter and subsequent processes.

Salmonellosis in sheep has been described by Australian (Josland, 1953) and New Zealand writers (Salisbury, 1958). Salisbury also emphasized the significance of stress and poor physical conditions on the incidence of salmonellae among flocks harbouring a small number of carriers.

Investigations have recently included an examination of horse meat and other prepared open pack pet foods. Of 72 samples of imported horse meat and pet food so far examined, 33 (45·8%) have been positive for a variety of salmonellae including

Table 5. *The incidence of salmonellae in horse meat and pet food*

Material	Origin	No. examined	No. containing salmonellae	Serotypes isolated
Horse meat	Argentine	13	11	*Salm. newport* *Salm. meleagridis* *Salm. reading* *Salm. anatum* *Salm. oranienburg* *Salm. bredeney* *Salm. nottingham* *Salm. havana* *Salm. rutgers* *Salm. mission* *Salm. cerro* *Salm. minnesota* *Salm. agodi*
	Rumania	15	6	
	Bulgaria	6	3	
	Poland	9	1	
	Hungary	3	0	
	'Imported'	9	6	
Pet food		17	6	
	Totals	72	33*	

* These constitute 45·8% of the total.

Salm. meleagridis, *Salm. oranienburg*, *Salm. newport*, *Salm. reading*, *Salm. anatum* and *Salm. bredeney* (Table 5). There is a flourishing import trade in frozen horse meat, some of which is advertised as fit for human consumption. Although these products may contain insufficient salmonellae to produce illness in cats and dogs, they will serve, nevertheless, to introduce salmonellae into the domestic kitchen. Further, the pets themselves may become temporary carriers and their excreta, near the house, will be a focus of infection for flies.

Pet foods and horse meat are sometimes sold in fish and meat shops which also sell food for human consumption, and the smaller retail shops still serve raw and cold cooked meats on the same counter, using the same weighing balance and sometimes the same utensils; the bigger shops are able to separate the raw and cooked meats and also to provide chilled and frozen storage facilities.

4. THE SIGNIFICANCE OF ANIMAL FEEDINGSTUFFS AND OTHER FOODS

The source of infection to the animals themselves is the subject of much discussion. There are many who think that modern feedingstuffs such as bone, blood and other high protein meals cause the infection. There is a wealth of evidence to show that high proportions of certain feedingstuffs are contaminated with small numbers of salmonellae (Report, 1959*a*, 1961). Table 6 (based on Report, 1961) shows the proportions of ingredients and complete feeds contaminated with salmonellae; it also shows that the heat of 'pelleting' destroys the majority of salmonellae. It has been suggested, also, that the animals may become infected by grazing in fields manured by artificial fertilizers such as bone meals contaminated with salmonellae, and that birds may foul the drinking troughs used by the animals.

Table 6. *The incidence of salmonellae in animal feedingstuffs*

(From Report, 1961)

Type of material examined		No. examined	Samples containing salmonellae	
			No.	%
Feed ingredients	Meat and bone meal	32	11	34·4
	Bone meal	8	1	12·5
	Fish meal	13	0	0·0
	Vegetable products	77	6	6·5
	Total	130	17	13·1
Finished feeds	Meals, mashes, etc.	67	6	9·0
	Pelleted foods	296	5	1·7
	Total	363	11	3·0
	Grand total	493	28	5·7

Few investigations have gone far enough to follow the passage of serotypes of salmonellae from feedingstuffs to animals or birds and from meat or egg products to the human cases of salmonellosis. The practical difficulties in producing such evidence are immense and the work can be carried out conveniently only in certain places: for example, the investigation by Newell *et al.* (1959) in Northern Ireland which described the occurrence of infections in farm stock fed with feeds contaminated with salmonellae.

Smith (1960) followed the course of events after feeding pigs free from salmonellae on food naturally contaminated with salmonellae. Post mortem examination was carried out on pigs killed at varying times after experimental feeding commenced. There was no ill health and no pathological lesions, but in some pigs salmonellae were found in mesenteric lymph nodes only, particularly after a long feeding period. Sometimes the faeces were positive, but not permanently so, and the lymph nodes all appeared to lose their infection.

Sojka & Gitter (1961) have published a detailed report on salmonellosis in pigs with reference to its public health significance and quoted several references to papers in which the writers assumed that the salmonella infections of calves, poultry and pigs came from feedingstuffs (Müller, 1957; Lerche, 1957; Schaal, 1957; Adam, 1957; Walker, 1957; and Newell *et al.* 1959). The authors pointed out that whereas the infection of pork and its products is due primarily to infected pigs, meat from uninfected carcases may become contaminated at various stages of preparation. It may be splashed with faeces, or bile from a punctured gall bladder, or it may be handled with contaminated equipment. It was also pointed out that the methods of cooking pork and its products, especially sausages and pies, may be inadequate for the destruction of salmonellae.

Whereas animal feedingstuffs can be heat treated to destroy infection it is difficult to think of any method of treatment which will rid meats of salmonellae, except indirectly by better hygiene on the farms and in markets and slaughterhouses and by reducing the time of travel between farms, market and slaughterhouses, and the waiting time before slaughter of animals.

The contamination of coconut (Galbraith *et al.* 1960) is not primarily an animal problem because the frequent isolation of *Salm. paratyphi* B indicates strongly that human carriers are responsible (Anderson, 1960*b*). Nevertheless, it is not impossible that animal carriers play their part when the nuts are drying for some weeks in heaps in the palm orchards before going to the mills. If the nuts are fouled on the outside by cattle grazing in the palm orchards, or by other animals or birds, the hands of the workers removing the green husks will become contaminated and the organisms could then be carried through to the kernel and added to the water stored in large tanks for washing the nuts. In this way several batches of kernels would be contaminated and the contamination carried over to the macerating machine and, by way of the hands of the workers, to the desiccated coconut drying on trays. The proposal to dip the kernels in boiling water before maceration and the use of smaller tanks and frequent changes of water for the washing of nuts may relieve the situation.

5. DISCUSSION

Reports on food poisoning in England and Wales for 1957–1959 (Report, 1958*b*, 1959*b*, 1960) show the rise in incidence of salmonellosis (Table 7). Report (1960) stated that the number of incidents of salmonellosis due to serotypes other than *Salm. typhi-murium* had increased more than threefold and that the proportion increased from 15% to 37% in the five years from 1954 to 1959. These 'other' salmonellae have been divided into endogenous (common in the United Kingdom before 1940) and exogenous types (unrecognized or uncommon before 1940). Many of the exogenous types have increased in incidence (Table 8) and it is thought that both human and animal foodstuffs may be responsible for this change. For example, all but 6 of the 39 different types from eggs and egg products have been isolated from human cases in the past 5 years, and all but 10 were isolated in 1959.

Table 7. *The incidence of food poisoning in England and Wales for the period* 1957–1959

(From Report, 1958*b*, 1959*b*, 1960)

Causal agents	Year			Total
	1957	1958	1959	
Salmonella spp.	4,218	4,841	5,038	14,097
Staphylococcus spp.	128	125	118	371
Cl. welchii	93	88	110	291
Chemical	1	0	0	1
Unknown	2,625	2,244	2,580	7,449
Total	7,065	7,298	7,846	22,209

Salm. thompson was also commonly isolated from human cases and from egg products. All of the 23 types from meat and meat products in 1959 were isolated from human beings in the preceding 5 years and all but one were isolated from human cases in 1959. *Salm. typhi-murium* was the commonest type in man and the commonest in both eggs and meat products. Of the 63 types from animal feedingstuffs and fertilizers, 47 have been found in human cases, but there was no predominance of *Salm. typhi-murium* or other type.

Table 8. *The increase in incidence of salmonella serotypes in England and Wales during the period* 1954 *to* 1959

(From Report, 1960)

Serotype	No. of incidents during the years		
	1954	1958	1959
Salm. newport	35	118	319
Salm. thompson	83	132	207
Salm. infantis	1	18	57
Salm. heidelberg	16	308	182
Salm. saint-paul	25	95	83

The phage typing of *Salm. typhi-murium* indicates that certain types are more able to become parasites in particular hosts; this may be true for other serotypes of salmonellae and would explain the predominance of certain types. There is no reason to suppose, however, that the more exotic types may not gradually become adapted to various hosts. Newell (1959) suggested that most salmonella serotypes when introduced to a susceptible animal population are propagated partly by animal to animal contacts and partly by the fortuitous spread of infection by man in feedingstuffs and by other means. There may be other types, however, that have a distinct host specificity, e.g. *Salm. dublin* in cattle and some of the exotic types in reptiles. The same author quoted investigations carried out on a farm using a feedingstuff contaminated with *Salm. infantis*, *Salm. orion*, *Salm. schwarzengrund* and other salmonellae (Newell *et al.* 1959). There was no illness among the stock but rectal swabs from 45% of the pigs tested were positive for one or other of these salmonellae and *Salm. schwarzengrund* was isolated from a caecal swab taken at the slaughterhouse. The hens became infected with *Salm. infantis* and produced eggs containing this organism. Pigs on other farms showed *Salm. orion* and it was assumed that *Salm. orion* was selectively taken up by pigs and *Salm. infantis* by poultry, and that certain animal species may provide a more favourable environment for survival and multiplication of a particular salmonella serotype than others. Furthermore, in Northern Ireland the first isolations of *Salm. orion*, *Salm. infantis* and *Salm. schwarzengrund* were made from human cases in 1958 after it had been confirmed that these types were being imported and were causing animal infections.

Newell suggested that *Salm. heidelberg* was introduced originally in a single batch of animal feedingstuff. It seems that it may now be endogenous in pigs. Galbraith, Archer & Tee (1961) suggested a similar history for *Salm. saint-paul.*

In 1932 Sir William Savage suggested that the habitat of salmonellae causing gastroenteritis was the animal kingdom, and that transmission by human carriers was rare. In 1956 he said that although much was known about salmonellosis in animals, more extensive surveys were needed to determine how frequently animals act as reservoirs of salmonella strains and how they are associated with food poisoning.

Co-operation between farmers, veterinary surgeons, doctors, diagnostic laboratories, public health authorities, meat inspectors, and slaughterhouse and canteen personnel is necessary. Each one of these is presented with a different aspect of the problem and a true picture will arise only from the collection of all experiences.

6. Summary

The carriage of salmonellae by animals and birds is considered in relation to the contamination of foods provided for human consumption. Egg products and raw meat are thought to be the commonest foods contaminated in this way.

The pasteurization of egg products and the pelleting of animal feedingstuffs will help to reduce the introduction of salmonellae into bakeries and farms.

Improved conditions and speed of transport for animals passing from farms to abattoirs and more care and attention over hygiene during processing may reduce the spread of infection among animals and the spread of contamination on carcase meat.

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THE SIGNIFICANCE OF VARIOUS ORGANISMS OF FAECAL ORIGIN IN FOODS AND DRINKING WATER

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1. GENERAL CONSIDERATIONS ON THE USE OF INDICATOR ORGANISMS FOR THE DETECTION OF CONTAMINATION OF FOODS

At a time when the detection of pathogenic species of the Enterobacteriaceae, especially salmonellae and shigellae, was a very difficult task, the use of so-called indicator organisms for these pathogenic types was the generally adopted and, in fact, the only possible procedure for the hygienic examination of drinking water and foods. The underlying idea was that if other bacteria belonging to the Enterobacteriaceae, but of similar resistance to unfavourable external conditions and more easily detectable, appeared to be absent from foods it would be very probable that the pathogenic types would not be present either. The indicator organism originally chosen by Schardinger (1892) was *Escherichia coli* because this organism is generally found in the intestinal contents of man and animals. A vast amount of research has been carried out since to develop methods for the detection of this organism in foods and drinking water. The special aspect of this type of test has always been the distinction of *E. coli* from *Aerobacter aerogenes* (Beijerinck) (an organism recently renamed *Enterobacter aerogenes*, Hormaeche & Edwards, 1960), because the latter was considered ubiquitous.

In view of the very many uncertainties still involved in the examination of food and water for so-called faecal pollution and especially because reliable methods are available nowadays for the direct detection of enteric pathogens in foods, the question whether there is still any place for the use of indicator organisms in modern food hygiene is warranted. The answer must be in the affirmative for the following reasons.

First, the direct detection of enteric pathogens in foods remains a dubious test because their distribution may be very uneven (Adam, 1956) and their isolation from an overwhelming majority (e.g. 10^6:1) of other food organisms is sometimes very difficult. This is especially so when organisms which have been weakened during a period under rather unfavourable conditions are to be recovered, because they do not generally survive a direct enrichment in the selective liquid media used at present.

Second, it should always be borne in mind that, apart from the classical enteric pathogens of the genera *Salmonella* and *Shigella*, other harmful organisms may be spread by food and many of these, especially the viruses and intestinal worms, can be detected either not at all, or only with rather complicated methods entirely outside the scope of the food microbiologist.

Third, whereas the proof of the absence of pathogens in a given sample of food has significance only for the sample under investigation, the repeated failure to find indicator organisms is potentially valuable as well because it is then very unlikely that food prepared in the same way will ever be dangerously contaminated (Mossel, 1958).

Finally, although questions of aesthetics must certainly not be confused with the protection of health, it is yet the task of the food hygienist to ensure that foods are marketed in an aesthetically acceptable form. Just as the discovery of sand in flour is not appreciated by the consumer, although this would not entail any risk to health, the presence in food of material which has been more or less recently shed by man or animal, is also quite unacceptable.

It is generally felt, therefore, that there is still a need for the use of indicator organisms in safeguarding man's food supply.

In the past particular attention was paid to micro-organisms which occur in human faeces. Today it is known, however, that the bacteria present in animal intestinal contents can be qualitatively and quantitatively different from those in humans. *E. coli*, for instance, although very frequent in man is rarely found in horses (Haenel, 1960; Kjellander, 1960). Also, the different species of Lancefield's group D streptococci do not occur to the same extent in the intestines of different animals (Winslow &

Palmer, 1910; Cooper & Ramadan, 1955; Buttiaux, 1958; Kjellander, 1960; Bartley & Slanetz, 1960; Kenner, Clark & Kabler, 1960). But from the point of view of hygiene all faecal contamination is equally dangerous, whether originating from domestic or wild animals, or from man. Pigs and birds are, for example, even more frequent carriers of salmonellae than is man (Hobbs, 1961).

A priori one should therefore consider as organisms indicative of faecal contamination of foods and drinking water all those which are normally present in considerable numbers in the human and animal intestine. Yet the application of this, in itself a sound principle, is hampered by the following limitations. First, it is only quite recently that serious attention has been paid to the microbiology of animal intestinal contents (Briggs *et al.* 1954; Wilssens & Buttiaux, 1958; Haenel, 1960; Smith & Crabb, 1961; Dickinson & Mocquot, 1961; Raibaud *et al.* 1961). As yet the influence of race, age, diet and other environmental factors has not been studied in detail, but it is already clear that in many cases the proportions of different bacterial species vary from one herd or flock of any given animal to another (Barnes, 1958) and even from one individual animal to the next; to a lesser extent the same seems to be true for man (Haenel, 1960; Emslie-Smith, 1961). Second, recent investigations have shown that the bacteria found in highest numbers in the human and animal intestine belong to the genera *Bacteroides*, *Bifidibacterium* and *Lactobacillus* (Misra, 1938; Seeliger, 1957; Braun, 1959; Mossel, 1959*b*). Because the two former genera comprise micro-organisms with a low resistance to extraenteral conditions (Mossel, 1959*b*; Gyllenberg, Niemelä & Sormunen, 1960) and members of the latter genus are ubiquitous, they all are of little use in practice.

2. CHARACTERS GOVERNING THE SUITABILITY OF CERTAIN BACTERIA AS INDICATOR ORGANISMS FOR FAECAL CONTAMINATION

To be suitable indicator organisms bacteria occurring in the intestine should possess the following properties. First, specificity; ideally the bacteria selected should occur only in intestinal environments. Second, they should occur in very high numbers in faeces, so as to be still encountered in high dilutions. Third, they should possess a high resistance to the extraenteral environment, the pollution of which is to be indicated. Fourth, they should permit of relatively easy and fully reliable detection even when present in very low numbers,

Among the bacteria considered by most public health bacteriologists to be suitable indicator organisms are found: coli-aerogenes bacteria and more particularly *E. coli*; faecal streptococci; and clostridia. The specificity, sensitivity and resistance of these groups of bacteria will now be considered and, *inter alia*, the properties of some other organisms normally present in the intestine.

(a) *Specificity*

(i) *Enterobacteriaceae*. The specificity of *Escherichia coli* (*E. coli* I, Report, 1956) is unquestionable, as the recent investigations of Buttiaux (1959) and Bartley & Slanetz (1960) have confirmed. That of other coli-aerogenes bacteria is still under discussion. Bartley & Slanetz quite often found 44°– *E. coli* in stools. In our opinion, organisms of the *Klebsiella* group, which Cowan, Steel, Shaw & Duguid (1960) define as being nonmotile Enterobacteriaceae, generally capsulated, V-P+, and urease+, are also specific indicators (Buttiaux, 1959). But this is not the case with *Citrobacter* spp. and species of *Enterobacter* (*Aerobacter aerogenes* and *Aerobacter cloacae*). The first are definitely found in faeces (Parr, 1936; Buttiaux, 1959) but also occasionally in soil which may only be slightly polluted or not at all. Our own investigations have so far revealed, however, that *Enterobacter* spp. occur only rarely in the human intestine.

It seems logical to attribute at least the same public health significance to coli-aerogenes bacteria which are lactose− or slow lactose fermenters as to the lactose+ types. Also, it is important to note that *Proteus* spp. occur consistently in human and animal intestinal contents, although normally in very low numbers (Haenel & Dawidowski, 1961).

(ii) *Group D streptococci*. Much work has been done on the specificity of these streptococci. They are nearly always found in human and animal faeces (Ostrolenk & Hunter, 1946; Ostrolenk, Kramer & Cleverdon, 1947; Briggs, Willingale, Braude & Mitchell, 1954; Barnes & Ingram, 1955; Barnes, Ingram & Ingram, 1956; Buttiaux, 1958; Bartley & Slanetz, 1960; Kenner *et al.* 1960). It is impossible, therefore, not to admit their faecal origin. That they are sometimes found in other environments (Buttiaux, 1951; Mundt, Johnson & Katchikian, 1958; Mundt & Johnson, 1959) cannot be denied, but this is due to their persistence and, as we will demonstrate, does not invalidate their usefulness as indicator organisms if the proper quantitative approach is made.

(iii) *Other groups of bacteria*. Of the other bacteria occurring in human and animal intestines, the specificity of *Bifidibacterium* and *Ristella* (*Bacteroides*) spp. is certain; *Clostridium perfringens* (*Cl. welchii*) is also found in a few other environments and lactobacilli in many others.

Bacteria which must not be overlooked in an up-to-date consideration of the suitability of indicator organisms are those of the type formerly called *Bacterium anitratum* and more recently *Acinetobacter anitratum* (Brisou & Prévot, 1954) but since the genus *Achromobacter* is now generally recognized as a nonmotile one (Shewan *et al.* 1960; Buttiaux, 1961), better indicated as *Achromobacter anitratum*. Papavassiliou (1955) and Wilssens & de Vleeschauwer (1960) found these bacteria in bottled milk and Mossel (1962) encountered them frequently and in quite high numbers (up to 10^3/ml) in bulked raw human milk. The ecology of these organisms is not yet quite clear (Billing, 1955). They are not encountered very often in faeces, but their frequent isolation from pathological material (Ferguson & Roberts, 1950; Lutz, Grootten, Velu & Velu, 1956; Ellis, 1961) makes their presence in foods still suspect.

(b) *Occurrence in high numbers*

The main groups in faeces, ranging from the most frequent to the least frequent, can be roughly listed in the following manner: *Bifidibacterium* spp., *Ristella* spp., lactobacilli, Enterobacteriaceae, group D streptococci and clostridia. As we have already said, important differences may occur between mammalian or avian species and within a species depending on physical condition, age and environment, especially the composition of the food or feed (Haenel *et al.* 1957, 1958) and the presence in it of antimicrobial factors.

The composition of the intestinal and faecal microflora in certain diseases has been studied by Thomson (1955*a*,*b*) and Weijers *et al.* (1960) in humans, while a similar situation may exist for various animals likely to be in a position to contaminate foods. The influence of age and especially diet on the enteric microflora of chickens has been demonstrated by Barnes (1958) and Elliott & Barnes (1959). Obviously the use of so-called medicated feeds (those containing antibiotics or other chemotherapeutics) entirely regulates the faecal microflora of the animals.

(c) *Response to the extraenteral environment*

As we have demonstrated (Mossel, 1959*b*; Buttiaux & Boudier, 1960), the behaviour of enteric and other indicator organisms under the conditions prevailing in water and foods entirely determines their suitability. Hence, of all the factors listed so far, this is the decisive one.

Among the organisms occurring in intestinal contents, resistance increases as follows: *Ristella* spp., *Bifidibacterium* spp., lactobacilli, *E. coli*, *Enterobacter* spp., *Klebsiella* spp., group D streptococci and clostridia. Resistance to extraenteral surroundings depends greatly, however, on the conditions prevailing in water and foods. In foods important effects are exerted by the amounts and types of minerals, organic substances and micro-nutrients; the pH and the nature of the organic acids present; osmotic pressure as determined by low moisture content or high concentrations of salt or sugars; the oxidation-reduction potential and poising capacity; the occurrence of antimicrobial factors; the nature of the heat-treatment to which a food has been subjected; and the temperature of storage (Mossel & Ingram, 1955).

It must be concluded that *E. coli*, the indicator organism universally used, is generally not as resistant as most pathogenic Enterobacteriaceae and is much less resistant than the viruses, the presence of which they should indicate. In dehydrated foods (Brown & Gibbons, 1950), shellfish (Kelly & Arcisz, 1954), food stored in a frozen condition (Burton, 1949; Larkin, Litsky & Fuller, 1955; Kereluk & Gunderson, 1959; Fanelli & Ayres, 1959; Raj, Wiebe & Liston, 1961) or under refrigeration (Mossel, 1961*b*), foods treated by ionizing radiation (Erdman, Thatcher & MacQueen, 1961) and waters subjected to various commonly used treatments (Gilcreas & Kelly, 1955; Kelly & Sanderson, 1958; Kabler, Clarke, Berg & Chang, 1961) *E. coli* often disappears, whereas various pathogens persist. In fact it is only in acid foods that *E. coli* may have particular value as an indicator organism, on the basis of the accepted resistance of this organism to lower pH values (Mossel & de Bruin, 1960). In general, however, *Klebsiella* organisms (Thomas, Druce & Elson, 1960; Mossel, 1962) and, of course, the frequently employed Lancefield's group D streptococci are of much more value as indicator organisms.

The situation may therefore be summarized as follows. The original investigators around 1890 chose *E. coli* as the indicator organism because of its general occurrence in high numbers in intestinal contents, a perfectly valid assumption which has since been repeatedly confirmed. They could at that time not know that the resistance of *E. coli* to extraenteral conditions was in many cases so much lower than that of enteric pathogens and hence that, in spite of their initial occurrence in high numbers, they would rapidly be outgrown by other organisms, sometimes including the pathogens. No reproach can be levelled at these original investigators other than that they were not prophets. The situation is different, however, for the public health bacteriologist of today; his choice of indicator organisms should be based on his more extensive knowledge and he should be prepared to make revisions in the future.

(d) *Ease and reliability of detection, enumeration and identification*

These are other very important factors, especially from the point of view of daily laboratory routine.

(i) *Coli-aerogenes tests.* The determination of the most probable numbers of coli-aerogenes bacteria in foods using enrichment methods in liquid media is of limited value because it has become increasingly apparent that the technique has three essential shortcomings: a very high coefficient of variation; a limited reliability due to bacterial antagonism (Habs & Langeloh, 1960) and synergism; and the impossibility of detecting anaerogenic types which nevertheless occur quite frequently (Thomas, Hobson & Elson, 1958).

It should be realized in addition that even the enumeration of coli-aerogenes organisms including *E. coli* on the lactose containing solid media generally used still has certain deficiencies. It may, first of all, lead to false positives, caused particularly by *Aeromonas* spp. In most cases these do not originate from the faeces of man or warm blooded animals (Lautrop, 1961). Fortunately *Aeromonas* spp. can be reliably and simply distinguished from Enterobacteriaceae by Kovacs' (1956) oxidase test (Buttiaux & Gagnon, 1959; Eddy, 1960*b*; Schubert, 1960; Steel, 1961).

On the same media, however, lactose− or slow lactose fermenting Enterobacteriaceae are often overlooked. There is no doubt that organisms such as the lactose− *E. coli* ('*Paracolobactrum coliforme*') have the same faecal origin and therefore the same public health significance as the typical *E. coli* and this is also the case for *Klebsiella* spp. (Buttiaux, 1959). When no direct tests for the presence of pathogenic, lactose− Enterobacteriaceae are carried out, which is very unfortunately quite often the case in so-called 'routine' bacteriological examinations of foodstuffs, the search for lactose+ bacteria only may lead to extremely dangerous, false conclusions. We have ourselves encountered a case in which a sample of egg white solids was passed by a public analyst's laboratory because it was negative in a coli-aerogenes test. We found, however, that it contained no less than about 10^4 salmonellae/g and at the same time confirmed that lactose fermenters were absent.

It is therefore much better to replace the use of lactose media by those containing mannitol, as suggested many years ago by Bulir (1907) and advocated again by Henriksen (1955) and Mossel (1957). Particularly recommended for this purpose is MacConkey's (1905) crystal violet-neutral red-bile agar ('violet-red-bile agar') to which 1% of D-mannitol is added. In this medium virtually all Enterobacteriaceae form visible colonies within 18 hr on incubation at 37°, whereas the growth of other bacteria is completely suppressed.

(ii) *Tests for* E. coli *I.* Although, as we have seen, a negative test for *E. coli* I gives no guarantee of the wholesomeness of a food, a test for this organism is still useful because, if it gives a positive result, the food sample under investigation must certainly be rejected. The recommended quantitative procedure here is a presumptive enumeration of these organisms by plating the food or a dilution of it on Chapman's (1951) tergitol-triphenyltetrazolium chloride (TTC) agar incubated at 44° (Buttiaux, Muchemblé & Leurs, 1953), followed by confirmation of a representative number of TTC non-reducing colonies by testing them with MacKenzie, Taylor & Gilbert's modification (1948) of Eijkman's test. If water is examined, or foods in which *E. coli* occurs sparsely, the primary plating on Chapman's agar must obviously be replaced by membrane filtration or enrichment of suitable quantities in brilliant green-bile broth (Buttiaux, Samaille & Pierens, 1956; Mossel, 1962).

(iii) *Tests for group D streptococci.* Various methods have been developed in the last few years for the enumeration of group D streptococci in foods (Barnes, 1956; Mossel, van Diepen & de Bruin, 1957) and in water (Slanetz & Bartley, 1957), as well as for their identification (Litsky, Mallman & Fifield, 1955; Leclerc, 1959; Kjellander, 1960). These organisms, therefore, may now be counted and confirmed by simple and reliable methods which can be carried out by every laboratory, however small.

(iv) *Simultaneous tests for several indicator organisms.* In view of the ecological facts given above, the examination of foods and water for the two groups of indicator organisms, Enterobacteriaceae and Lancefield's group D streptococci, gives much more reliable results than a test for *E. coli* alone. Because satisfactory methods are available for the detection of both groups of indicator organisms, their simultaneous enumeration is definitely recommended for the estimation of the hygienic quality of drinking water and foods (Buttiaux, 1959).

To extend these tests so as to include also the enumeration and identification of sulphite reducing clostridia is not too difficult, because a reliable method for this exists (Mossel, 1959*a*). However, the results so obtained have only limited ecological value because, as indicated before, sulphite reducing clostridia found in foods may not always be of faecal origin: but this does not detract from the fact that if such clostridia are found in significant numbers (e.g. well over 10^2/g) an undesirable bacteriological condition is indicated, because, quite apart from faecal contamination, it points to incipient spoilage and/or health risks, namely an actual or potential incidence of *Cl. perfringens* food poisoning (Hobbs *et al.* 1953) and, more rarely, botulism.

3. The Significance of the Numbers of Micro-Organisms of Faecal Origin in the Interpretation of the Hygienic Quality of Water and Foods

There has been much confusion in the past about the interpretation of the results obtained in the examination of food samples for the presence of indicator organisms. One of the reasons for this confusion was that many investigators have demonstrated the presence of *E. coli*, faecal streptococci and similar organisms in a great number of sound foods, just by enriching sufficiently large quantities. It is striking to note that as early as 1895 von Freudenreich issued a warning in this respect and suggested means of overcoming these difficulties, namely by a quantitative approach to the subject. It is neither of importance nor significance to report that a food 'contains'

E. coli etc., but only a statement of the number of viable organisms found /g or /ml is warranted, preferably with at the same time an indication of the approximate variation of the count, or at least an indication of whether the figure was obtained by plate or by dilution counts (Buttiaux & Mossel, 1957).

When it comes to the interpretation of such figures, it is important to know that in most cases faecal pollution of foods at the point of origin is slight. One can say that direct faecal contamination does not often exceed 25 mg of faecal matter/10 kg of food, which in turn gives rise to a maximal density of 100 Enterobacteriaceae, 10 group D streptococci and slightly fewer clostridia/g of food. These figures are certainly never reached in practice, at least not with *E. coli*, because Borneff (1953) has demonstrated that a rapid decline in numbers of these organisms occurs when they remain on hands or equipment. Only in a few special cases, where foods are prepared in a particularly insanitary way, foci of infection existing in badly cleaned equipment may contaminate small amounts of the products right from the beginning at a level well in excess of 10/g (Abrahamson & Clinton, 1960).

The fate of this, generally very slight, initial contamination depends, as discussed above, entirely on the environment to which the bacteria are exposed. Therefore it is necessary to consider water and foods separately.

(i) *Drinking water*. In water the indicator organisms, which are mainly heterotrophic, have little or no chance of multiplying: on the contrary, they are quite often progressively destroyed (Buttiaux & Boudier, 1960). The occurrence, therefore, of a single organism specific for faecal contamination in, for example, 100 ml of untreated water, is still very significant.

Waters treated by chlorination or ozone should be considered separately. In these waters the removal of *all* Enterobacteriaceae is imperative since their sensitivity to the bactericidal agents used is virtually the same as that of salmonellae and shigellae (Kabler & Clark, 1960). Group D streptococci and particularly spores of clostridia are much more resistant however, and tolerate the usual treatment; relatively low numbers of these organisms have therefore to be accepted.

(ii) *Foods*. In most so-called perishable foods, that is those showing a pH of over 4·5, bacteria of faecal origin will multiply more or less actively unless unfavourable conditions prevail due to treatments such as condensation or dehydration or storage at reduced temperatures. Numbers of these organisms in foods at levels well over 10/g thus indicate bacterial growth in the commodities under investigation, due to faulty storage conditions or preparation, the latter including the exceptional case outlined in the beginning of this Section.

This quantitative approach makes it also very easy to deal with the frequently voiced objection to the use of Lancefield's group D streptococci as indicators of faecal contamination of foods because of their relatively high resistance to unfavourable conditions like freezing, desiccation (Buttiaux, 1951) and heat treatment (Shattock, 1955). This objection must of course be admitted and so must the possibility that the few streptococci present in sound raw materials like milk and meats will survive many of the terminal heat treatments applied to foods in practice. However, if in a sample of a heat treated food group D streptococci are found in numbers well over 10^2/g, this suggests one of the three following undesirable features:

the raw material was rather seriously contaminated; the foods were subjected to severe contamination after adequate pasteurization; or the foods were stored under conditions permitting growth of the few group D streptococci which might be present in sound foods immediately after a heat treatment.

There is one possible exception to this rule, namely the occurrence of the so-called psychrotrophic Enterobacteriaceae (Mossel & Zwart, 1960; Eddy, 1960*a*), which are able to multiply in refrigerated foods. However, because the growth curves of the various psychrotrophic bacteria vary considerably the speed of development at about 5° of every strain isolated should be determined before it is concluded that observed high counts are due to the incidental occurrence of psychrotrophic strains (Mossel, 1961).

For a sound interpretation of counts and types of bacteria of faecal origin it is further imperative to distinguish between two categories of food, namely raw and heat treated commodities. In the former a maximum of 10 *E. coli*/g of food will be tolerated whereas the numbers of other non-pathogenic Enterobacteriaceae may be slightly higher (10^2/g) because they may well belong to the natural flora of the commodity. In heat treated foods, however, as in treated waters, not only must there be fewer than 1 *E. coli*/g but other Enterobacteriaceae, and in fact virtually all Gram-negative rods (including *Aeromonas*, *Pseudomonas*, *Achromobacter*, *Flavobacterium* and *Xanthomonas* spp.) must not exceed this number, because significant numbers of any of these bacteria indicate either insufficient heat treatment or subsequent contamination (Mossel, 1956).

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(Symposium on Bacteria of the Intestine: Contribution relating to Paper IX)

THE SAFETY AND CLEANLINESS OF WATERS AND FOODS

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SUMMARY: There are two reasons for the bacteriological examination of water supplies and foods; first to determine whether the commodity is safe to consume, i.e. that pathogenic organisms are absent; second, to determine whether the commodity is clean, i.e. that the level of nonpathogenic organisms is such that the commodity will suffer no significant deterioration in quality during normal conditions of storage and transport between preparation and delivery to the consumer. In the examination of water supplies and of shellfish growing in polluted waters, both the safety and the cleanliness of the product are assessed by a single examination for the presence of the normal inhabitants of the intestinal tract of man and animals. In the examination of foods only the cleanliness of the product is assessed by an examination for the presence of normal intestinal inhabitants. The safety of foods can be assessed only by examination for the presence of pathogenic organisms.

The primary purpose of the bacteriological examination of water supplies is to ensure their freedom from human excreta and therefore from the organisms of intestinal infections peculiar to man. The intestinal infections peculiar to man, transmitted by the faecal contamination of water supplies, are typhoid and paratyphoid fevers, the dysenteric infections and cholera.

In human intestinal infection, in both acute cases and in the carrier state, pathogenic organisms in the excreta often outnumber the normal inhabitants of the intestinal tract (Thomson, 1954, 1955). However, as at any time the number of acute cases and carriers of intestinal infection never forms more than a small proportion of the population, in sewage the normal inhabitants of the intestinal tract far outnumber the pathogens present. Of the normal inhabitants of the intestinal tracts of man, *Escherichia coli*, the most chacteristic organism of human faeces, is commonly present in numbers up to 5×10^8/g (Thomson, 1955).

The choice of *E. coli* in water examination as an indicator of recent contamination with sewage is based on the following facts: it is invariably present in human excreta; its numbers exceed those of group D streptococci and clostridia in human excreta; it survives but does not multiply in water; its time of survival in water is shorter than that of other organisms normally present in excreta; and its time of survival in water approximates to the time of survival of human intestinal pathogens. Evidence of remote sewage pollution of a water supply is afforded by the presence of 'presumptive coli-aerogenes organisms', group D streptococci and clostridia, associated with the absence of *E. coli* and hence of pathogens also. The presence of *E. coli* in a water supply is, therefore, the most sensitive indicator of recent faecal pollution and thus of the potential presence of pathogenic organisms. With one exception—a water contaminated solely by the urine of a urinary carrier of the typhoid organism—the cleanliness and therefore the safety of a water may be assessed by an examination for the presence of *E. coli*. *E. coli*, however, does not occur exclusively in human excreta but is also found, though in smaller numbers, in the excreta of animals and birds. An opinion as to the source of *E. coli* in water cannot be given by bacteriological examination, but only by a topographical survey of the supply to determine the sources of pollution. If the source of *E. coli* in a water supply proves to be a dead sheep in a tributary in a catchment area it is obviously less significant than if the source is the untreated crude sewage of a caravan site.

Pollution of a water supply with human excreta is always of greater significance than pollution with animal excreta, because animals are not infected by, nor do they harbour, the organisms of typhoid fever, paratyphoid fever, dysentery or cholera. Although animals act as hosts to a wide range of serotypes of salmonellae capable of causing infection in man, there is no evidence of an animal carrying typhoid or paratyphoid, dysentery or cholera. Typhoid fever, because of the small numbers of organisms necessary to produce infection in man, is the classical water borne infection in Western civilization. A water supply recently polluted with human excreta must always be considered a potential source of typhoid fever.

The use of *E. coli* as an indicator of recent pollution with human excreta and therefore of the potential presence of human intestinal pathogenic organisms is restricted to water and to shellfish growing in polluted waters. Furthermore, as *E. coli* does not multiply in water or in shellfish, the numbers present afford an indication of the degree of pollution. With foods, the position is different. Under suitable conditions of temperature, foods form excellent media for the growth of organisms; the number of organisms present at the time of examination may therefore afford no information about either the extent or the source of the original contamination. The number of organisms present in raw milk, liquid egg, prepared meat products, or raw fish at the time of delivery to the consumer may reflect the conditions of preparation, storage and transport, rather than the extent of the original contamination.

The significance to be attached to the isolation from water of normal intestinal inhabitants does not extend to foods. The presence of *E. coli* in a raw food, for example, cannot be regarded as evidence of recent pollution with human excreta, nor can its presence in a food subjected to heat treatment be regarded as evidence of direct pollution with human or animal excreta. In addition, the absence of *E. coli* from a food cannot be regarded as an indication that intestinal pathogenic organisms are also absent. The flesh of an animal slaughtered in the septicaemic stage of a salmonella infection, or the meat of an egg laid by a hen with an ovarian focus of salmonella infection, may contain large numbers of salmonellae but no normal inhabitants of the animal intestine.

Although few foods are free from one or other of the normal inhabitants of the animal intestine, in many the association of these organisms with pathogens is erratic and unpredictable. Thus Hobbs & Wilson (1959) found that high colony and coli-aerogenes counts on imported carcase meat did not necessarily indicate salmonella contamination, whereas, conversely, salmonellae were found in samples in the absence of *E. coli* and in samples with low colony counts. Galbraith, Hobbs, Smith &

Tomlinson (1960) reported similar findings with imported desiccated coconut. The freedom from pathogenic organisms of foodstuffs cannot, therefore, be determined indirectly as in the examination of water and shellfish. The safety of a foodstuff can be assessed only by examination for the presence of pathogenic organisms.

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SOME OBSERVATIONS ON COLI-AEROGENES BACTERIA AND STREPTOCOCCI IN WATER

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1. Introduction

The paper by Dr. Buttiaux and Dr. Mossel concerns food and water. This contribution concerns solely water and not food, bearing in mind that the same reasoning does not apply to the two. The main difference between these two aspects is that, although organisms of possible faecal origin can multiply in some waters in some circumstances, water is not such an effective medium as many foods. The difference between animal and human intestinal organisms and between different animals has been stressed with a view to the possible differentiation between human and animal pollution. There is also variation between different races and different parts of the world. This may be due partly to differences in diet and partly to climatic differences. Wilson's Irregular Type VI of the coli-aerogenes group illustrates this point well. In this country it is relatively rare both in faeces and in water, whereas in many tropical countries it is relatively common in both these sources and has in consequence a different sanitary significance.

To attribute the same public health significance to coli-aerogenes bacteria that are lactose negative or slow lactose fermenters as to the lactose positive varieties, raises practical difficulties of determining specificity. Slow lactose fermenting coli-aerogenes bacteria are very commonly found in nonfaecal sources. Salmonellae are also lactose negative but they are not specifically sought in water, and their absence, at least from water, is inferred from the absence of *E. coli*. The same would apply to lactose negative *E. coli* strains. Since the adoption of membrane filtration for the routine examination of water it has become apparent that in some waters bile tolerant organisms which do not ferment lactose greatly outnumber the lactose fermenters and that many of the lactose fermenters are anaerogenic.

2. THE TEMPERATURE PREFERENCES OF COLI-AEROGENES BACTERIA

The coli-aerogenes group is normally differentiated by IMViC typing, but it is doubtful whether this is of any value in assessing the sanitary significance or possible faecal origin of organisms other than *E. coli* I. The same IMViC types as are found in faeces can be isolated from a variety of nonfaecal sources. In an attempt to throw more light on the sanitary significance of this group a study of the temperature relationships of coli-aerogenes bacteria isolated from a variety of sources has been started. This contribution is in the nature of an interim report on the progress of this work.

Typical *E. coli* I grows at relatively high temperatures, up to 46°, but much larger numbers of coli-aerogenes organisms can be isolated from soils and water at relatively low temperatures (30°) than at 37°. In the present study coli-aerogenes bacteria are being isolated from a variety of sources at 30° and 37° and their growth characteristics studied at temperatures from 30° to 42°. Isolations are being made from faeces, sewage, sewage effluents, rivers and subsoils underlying water pipes. The 48 hr presumptive positive MacConkey's broth tubes from routine deep well samples and organisms from persistently positive samples from new and repaired mains are also being studied. Cultures producing acid and gas, even if only in minimal detectable quantities, from MacConkey's broth at some point in the temperature range 30–42° are being included in the survey.

To determine optimum temperatures, subcultures are made from 18 hr peptone cultures into 5 MacConkey's broth tubes, 1 of each set being incubated at 30°, 33°, 35°, 37° and 42°. Quantities of acid and gas are recorded daily up to 3 days, a scoring of 1 to 4 being used for each. Optimum conditions are taken as the highest product of these two figures in the shortest time. All cultures showing no acid or no gas are thus recorded as negative. Many cultures grow equally well at several temperatures and in Table 1 these have been recorded several times under the heading 'optimum growth'. The maximum temperature at which optimum growth occurs is also being abstracted, as this avoids duplication and gives a clearer picture.

The number of cultures in some groups is as yet small, but some indication of trends can already be seen from Table 1. Cultures from faeces show a marked preference for temperatures over 35° with very few negatives at any temperature. Sewage effluent and river water cultures show preferences more evenly distributed over the whole temperature range, with a slight preference for the lower temperatures and a high proportion of negatives at 37° and 42°. In the soils there is an even greater frequency at the low temperatures, with none preferring 42° and a very high proportion of negatives at 37° and 42°. The few cultures isolated so far from sewage have more the characteristics of the soil cultures than the faeces cultures.

From these results, therefore, there would appear to be a fairly rapid replacement of organisms with a high temperature preference by those with a lower temperature preference, through the dying out, or changes in characteristics, of the high temperature organisms or the multiplication or influx of low temperature organisms. It will be noted that faeces yielded a few cultures with a low temperature optimum: therefore organisms with this characteristic cannot certainly be nonfaecal in origin, although their chances of having a faecal origin are less than those with higher temperature optima possess.

Table 1. *The growth temperature characteristics of coli-aerogenes bacteria from various sources*

Source and no. of cultures	Temperature characteristic†	Nos. of cultures with given characteristics				
		30°	33°	35°	37°	42°
Faeces 33	(a)	5	11	16	23	12
	(b)	1	3	5	12	12
	(c)	1	1	1	2	2
Sewage 8	(a)	5	5	4	1	0
	(b)	2	2	3	1	0
	(c)	0	0	1	3	8
Sewage effluent 21	(a)	13	13	11	8	6
	(b)	4	5	4	2	6
	(c)	0	0	1	7	13
Rivers 25	(a)	13	15	13	10	3
	(b)	6	5	4	7	3
	(c)	1	0	2	6	11
Soils 21	(a)	14	14	8	1	0
	(b)	5	8	7	1	0
	(c)	0	0	1	12	18
Deep wells* 25	(a)	8	14	14	4	0
	(b)	1	8	12	4	0
	(c)	0	0	0	3	21
Repaired mains 11	(a)	6	5	7	6	1
	(b)	1	1	3	5	1
	(c)	0	0	0	0	5

* Presumptive positive tubes in 48 hr. †Temperature characteristics of growth: (a), optimum; (b), maximum temperature at which optimum growth occurred; (c) no growth.

The 48 hr presumptive positive cultures from deep wells more closely resemble the soil cultures than any others, with no cultures preferring 42° and nearly all negative at 42°. They have a slightly higher temperature preference than those from the soils; but as all these cultures were originally isolated at 37° there was some initial selection. It would be fairly safe to assume from these results that these organisms have little if any sanitary significance.

The cultures from mains were from new or repaired mains that had persisted in giving coli-aerogenes organisms on two or more occasions. They were isolated at 37° in 24 hr or less; there was, therefore, again some initial selection. Numbers are still too small to permit a reliable indication, but preference seems to be for 35–37° with a marked dislike of 42°. This does not exclude the possibility of faecal origin. Although differentiation by this means is not clear cut it seems likely to provide more reliable evidence of faecal origin than IMViC typing.

3. THE MULTIPLICATION OF COLI-AEROGENES ORGANISMS IN WATER

The ability of coli-aerogenes organisms to multiply in some extraenteral environments has been quoted. Evidence of their very considerable multiplication in special circumstances has been obtained. The growth of algae in reservoirs is often controlled by treating the water with copper sulphate, and the Queen Mary reservoir in the Thames Valley is normally plagued with a spring growth of diatoms which can interfere with subsequent filtration. This can be prevented or destroyed by treatment with copper sulphate at a concentration of 0·5 to 0·75 mg/l., but in 1958 no copper treatment was applied because extensive additional filtration area was available following the installation of Ashford Common Works. In 1959, because of greater demands, partial copper treatment was carried out on April 1st using 5 tons of copper sulphate over the outlet area, equivalent to 0·5 mg/l. In the autumn of 1959, following a very hot dry summer, an extensive growth of *Microcystis* occurred in the reservoir. To avoid a recurrence of this the reservoir was treated on March 28th, 1960 with 23 tons of copper sulphate over the whole reservoir (equivalent to 0·75 mg/l.) and on March 23rd, 1961 with 15 tons (equivalent

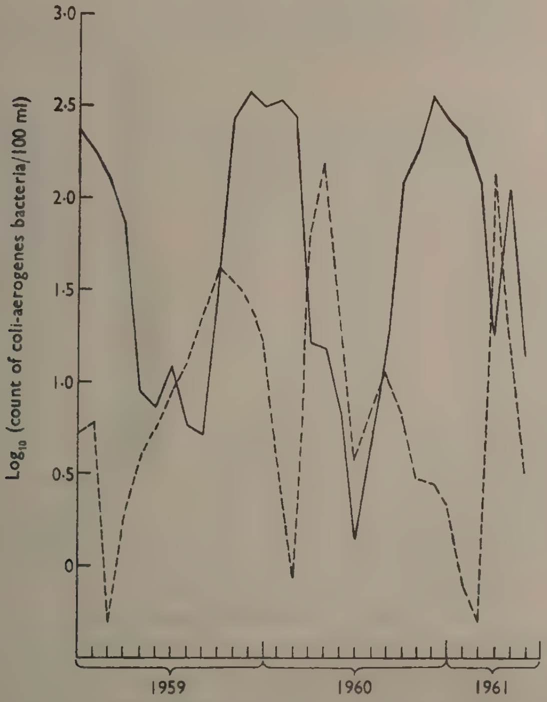

Fig. 1. The variations in the monthly mean counts of coli-aerogenes bacteria in water from the Kempton Park Works during the period 1959–1961. Continuous lines, reservoir water; broken lines, filtered water.

to 0·5 mg/l.). Another 15 tons is being kept in reserve should there be any excessive multiplication of *Microcystis*.

The interesting feature of all this from the point of view of this discussion is the effect of the treatment on the coli-aerogenes bacteria. This can best be seen from a study of the monthly mean counts of coli-aerogenes bacteria in the reservoir water and the filtered water from the Kempton Park works. From the graphs in Fig. 1 it will be seen that the coli-aerogenes count on the stored water as it reaches the works shows a pronounced seasonal variation, being highest in winter, falling sharply in the spring, remaining low in summer, and rising again in the autumn. The count on the filtered waters follows a different pattern, with very marked minima in March and high counts in September-November. In 1959, with very little copper treatment of the reservoir, the rise and fall from March 1959 to March 1960 was very regular, but in 1960 and 1961, following heavy dosing with copper at the end of March, the coli-aerogenes counts on the filtered water rose well above those given by the reservoir water. This occurred on both occasions very dramatically after 'coppering' and persisted for some weeks. This response is confined to the coli-aerogenes organisms, there being no corresponding increase in *E. coli*.

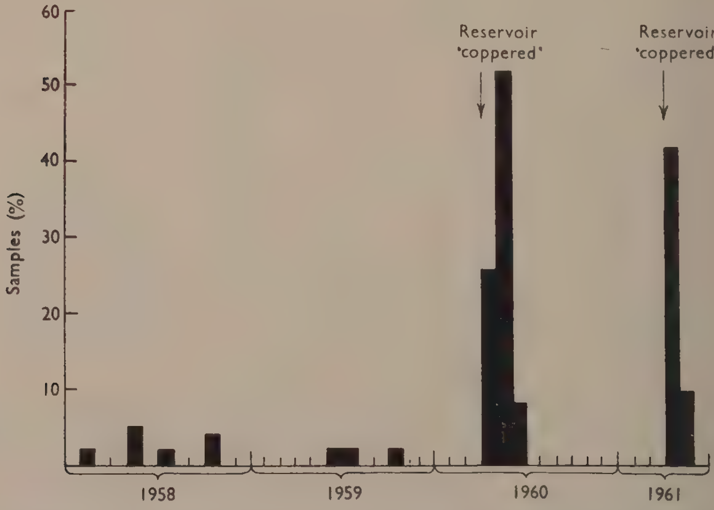

Fig. 2. The incidence of samples of Kempton Park filtered water containing coli-aerogenes bacteria in excess of 50/100 ml and *E. coli* less than 10/100 ml during the period 1958–1961.

This effect shows up even better (thus enabling conditions at different works to be more closely compared) when the frequency distributions of samples giving coli-aerogenes counts over 50/100 ml with *E. coli* counts under 10/100 ml are studied. These are shown as histograms in Figs. 2 and 3 for Kempton Park and Hampton Works, respectively. A few such samples occurred sporadically at other seasons and in years without 'coppering' but to nothing like the same extent as following 'coppering'. In 1960 high results first occurred 25 days after 'coppering' and persisted for 7½ weeks, and in 1961 the effect showed 21 days after 'coppering' and persisted for 3 weeks. The highest coli-aerogenes count recorded was 760/100 ml. At Hampton Works, which receives half Queen Mary water and half from another reservoir, this phenomenon does not occur. No IMViC typing has been carried out on these organisms, their optimum temperature relationships have not been determined, and no special experimental work to elucidate this phenomenon has as yet been conducted. These are merely observations on results obtained for routine works control purposes.

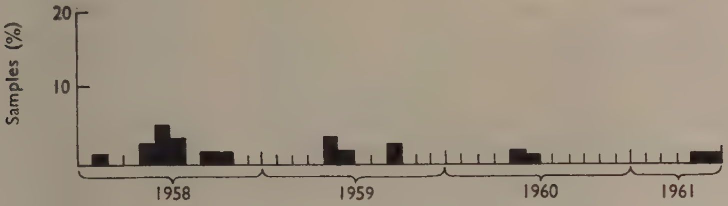

Fig. 3. The incidence of samples of Hampton filtered water containing coli-aerogenes bacteria in excess of 50/100 ml and *E. coli* less than 10/100 ml during the period 1958–1961.

It is apparent that these organisms are multiplying in the filter bed and their sanitary significance must therefore be regarded as negligible. The cause of this increase is another story. As no parallel increase occurs in the reservoir water, it is justifiable to assume that they are not growing on the dead algae. Contrary to opinions sometimes held, the copper is not all precipitated as carbonate in the reservoir. This is a slow process and some copper (of the order of 0·25 mg/l.) passes on to the filter beds. This could kill some of the algal or other growths in the filter skin, thus providing a fairly concentrated growth nutrient for the coli-aerogenes bacteria, or the copper could possibly interfere with the activities of organisms antagonistic to coli-aerogenes bacteria. At Hampton the copper is diluted with other water; also it passes through a small subsidiary reservoir where further precipitation of copper takes place. The whole phenomenon requires detailed investigation: but whatever the cause, it affords a very good example of the extraenteral multiplication of coli-aerogenes organisms other than *E. coli*, and indicates the unsatisfactory nature of this group of organisms as indicators of faecal pollution.

4. The Relative Survival of Faecal Streptococci and Coli-Aerogenes Bacteria

According to Buttiaux & Mossel, group D streptococci and, particularly, spores of clostridia are much more resistant than Enterobacteriaceae, and tolerate the usual water treatment processes. Relatively low numbers of these organisms have thus to be accepted in a supply. This is undoubtedly true of clostridial spores, these being quite frequently present in treated waters, particularly those from polluted wells which are unfiltered and treated solely by chlorination and dechlorination. But even these waters would not be expected to contain group D streptococci. To make a quick check on this statement 34 samples from two such wells, which normally yield clostridial spores, have been examined for streptococci in 100 ml samples over the past few weeks, with completely negative results.

There is evidence, however, that group D streptococci are more likely to survive than the coli-aerogenes group (particularly *E. coli*) under a variety of natural or less drastic conditions. As further evidence of this greater potentiality for survival, the results of analyses of samples from two shallow wells and from newly laid mains obtained between July 1953 and May 1961 have been examined in detail and are shown in Table 2. Faecal streptococci were counted by a multiple tube method using glucose-azide broth incubated at 37° for 3 days, followed by subculture, as soon as positive results appeared, to the same medium at 45° for 2 days. From July 1953 to December 1955 coli-aerogenes counts were done in MacConkey's broth by incubation at 42° for the first 18 hr, followed by 37° for the next 6 hr, with confirmation of *E. coli* in brilliant green-bile broth at 44°. From January 1956 primary incubation was at 37° throughout. It can be deduced from Table 2 that the lower incubation temperature in period (b) led to marked increases in the percentages of samples containing coli-aerogenes organisms other than *E. coli*, most of these being unaccompanied by *E. coli* or streptococci (especially in the mains). In the well samples there were more showing *E. coli* and no streptococci than *vice versa*. In the mains samples, however, the reverse was the case, there being far more samples showing streptococci but no *E. coli* than *vice versa*. In all cases it was relatively uncommon to find streptococci in the absence of any of the coli-aerogenes group, especially when the lower incubation temperature was used.

Table 2. *The incidence of coli-aerogenes bacteria and streptococci in shallow wells and mains water*

Source of samples	Sampling period* and no. of samples†	Samples (%) containing				
		Streptococci			No streptococci	
		Coli-aerogenes bacteria		No coli-aerogenes bacteria	Coli-aerogenes bacteria	
		E. coli	No *E. coli*	No *E. coli*	*E. coli*	No *E. coli*
Shallow wells	(a) 125	43	14	10	16	17
	(b) 361	35	14	3	21	27
New mains	(a) 162	5	18	2	7	68
	(b) 556	2	12	1	3	82

* Sampling periods: (a), July 1953–December 1955; (b), January 1956–May 1961.
† Samples negative for both coli-aerogenes and streptococci have been omitted.

These results show that in the shallow wells there is a tendency for the streptococci to outlive *E. coli*. If this were not so the percentage of samples with streptococci but no *E. coli* would be expected to be much lower. In the mains samples, which were contaminated presumably during laying by soil and dirt which penetrated the joints where organisms were relatively inaccessible to subsequent treatment with chlorine, the streptococci have shown an even greater potentiality for survival. This, however, does not indicate ability to survive normal adequate chlorination, but a greater ability to survive under marginal conditions, and the presence of occasional streptococci in the supply would not be accepted as a normal condition.

The results from these two groups of samples were further examined by calculating median ratios of *E. coli*:faecal streptococci. In the case of the 180 shallow well samples containing both these organisms the ratio was 1·4:1 and for the 20 mains samples it was 1·5:1. A recent batch of 59 raw river water samples examined over the last few weeks by the same subculture procedure has given a ratio of 17·5:1. These results again suggest the greater potentiality for survival of faecal streptococci with remoteness from the source of pollution.

5. THE APPLICATION OF MEMBRANE TECHNIQUES FOR COUNTING FAECAL STREPTOCOCCI

Various workers during the last few years have used improved methods of counting faecal streptococci in water, including some membrane filtration techniques (Slanetz & Bartley, 1957; Colobert & Morelis, 1958; Kenner, Clark & Kabler, 1961), and others have suggested new techniques for differentiating streptococci of human and animal origin (Cooper & Ramadan, 1955; Barnes, 1956*a,b*; Spaander & Roest, 1959; Bartley & Slanetz, 1960).

Using various modifications of these media with membrane filtration for raw River Thames and Lea waters and shallow wells, very high proportions were found of streptococci which did not conform to any known type. Dr. Barnes kindly examined a selection of these for us and confirmed that they were not groups D or N, nor *Strep. lactis*, *cremoris* or *equinus*. These organisms were relatively common in the waters examined, whereas typical *Strep. faecalis* was relatively uncommon.

For these isolations the membrane cultures were incubated at 37° for 48 hr. If, however, the membrane cultures were incubated for the first 4 hr at 37° and for the next 44 hr at 45°, the method was much more selective and the organisms isolated could more readily be assigned to recognized group D species.

Atypical streptococci can also be isolated from the presumptive glucose-azide broth tubes of the multiple tube test at 37°. Most of these cultures which failed to confirm at 45° were streptococci, but no attempt was made to classify or identify them further as failure to grow at 45° was regarded as evidence of nonfaecal origin. In the above series, 81·7% of 1,064 positive presumptive azide tubes at 37° from shallow wells confirmed at 45°; 78·8% of 500 tubes from new mains confirmed at 45°; and 78·5% of 432 tubes from river water confirmed at 45°. These atypical strains thus seem fairly equally distributed in all these classes of water supplies.

6. SUMMARY

Work in progress tends to support the view that differentiation of the coli-aerogenes group by determining temperature preferences gives more useful information than IMViC typing. An example is given of the multiplication of coli-aerogenes organisms

in water, providing further evidence of the unsuitability of this group other than *Escherichia coli* for assessing sanitary significance. Evidence is submitted of the relatively greater ability of faecal streptococci than *E. coli* to survive in various natural and antagonistic environments. but the tolerance of these organisms in a chlorinated supply is challenged. To avoid isolating a large proportion of atypical streptococci from raw water, a membrane method is advocated involving incubation for the first 4 hr at 37° followed by 44 hr at 45°.

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The Editors of The Journal of Applied Bacteriology *do not accept responsibility for the Reports of the Society's Proceedings. Abstracts of papers read are published without verification of the scientific content. As a rule where no abstract follows the title the paper read has formed the basis of a full length paper in the* Journal.

THE SOCIETY FOR APPLIED BACTERIOLOGY

Winter Meeting

The Winter Meeting was held in the Barnes Hall of the Royal Society of Medicine, 1, Wimpole Street, London, W.1, on Wednesday, 11 January, 1961. In the morning there was a short business meeting, followed by a paper reading session. In the afternoon there was a Joint Meeting with the Microbiology Group of the Society of Chemical Industry, when the subject under discussion was 'Quantitative Microbiology'.

Morning Session. The following papers were read:—

Some Speculations on the Rideal-Walker Test. By R. B. Morrison (*Bacteriology Department, Anderson College, University of Glasgow*).

A suggested programme of investigations is given with a view to improving the validity of the Rideal-Walker test while at the same time maintaining its simplicity. The view is held that there is a need for a simple test of disinfectant activity which should be met despite the limitations and inadequacies of any such test. The main changes suggested are: first, the replacement of *Salmonella typhi* by *Escherichia coli* and the inclusion of a Gram-positive organism; second, the determination of a coefficient with and without the presence of organic matter; third, the substitution of a dropping pipette for the platinum loop; and fourth, the replacement of Rideal-Walker broth by a modern dehydrated culture medium.

The Production of Sugar for the Canning Industry. By J. A. Watson (*Tate & Lyle Ltd., Liverpool*).

Sugar produced specially for the canning industry must conform to specifications for the counts of thermophilic bacteria laid down by Gillespy at the Campden Fruit and Vegetable Research Station.

Normal granulated sugar may be outside these standards and various techniques have been tried to produce sugar of very low thermophilic count: selection, filtration while the sugar was in the liquid phase, and treatment with X-rays and cathode rays were all either only partially successful or impracticable to carry out. However, it was found possible to achieve counts well within specification, and often complete freedom from thermophiles, by the use of ultraviolet light. A plant was designed to use this process and consists of a battery of fixed germicidal striplights separately enclosed by rotating metal drums all fixed at an angle of a few degrees to the horizontal. Sugar for treatment is fed in at the high end of each drum and passes down it in a continuous curtain; the time of contact with ultraviolet light can be varied by varying the angle of fall. Various safety devices are built in to ensure that there is no danger of contamination by glass should a lamp break. The failure of a lamp without breaking will also bring a cut out into operation.

The Use of Selective Media for Counting Soil Micro-Organisms. By JANE MEIKLEJOHN (*Rothamsted Experimental Station, Harpenden, Herts*).

The total number of micro-organisms in a gramme of fertile soil is enormous, and attempts to count them all are not likely either to be successful or to yield any useful information. The use of selective media, however, enables those few thousands or hundreds of particular interest to be separated from the other hundreds of millions. For example, much information can be obtained about the bacteria concerned in the nitrogen cycle. Species of *Azotobacter* and *Beijerinckia* can be detected and counted by Jensen's method, which involves the surface inoculation and short incubation of agar plates containing a small amount of glucose and a trace of molybdate, but no added nitrogen. The important details of Hart's method for anaerobic nitrogen fixers are: that the medium should contain sugar, molybdate, and yeast extract; and that the tubes should be incubated in an atmosphere containing N_2 and CO_2 only. Details are equally important for the dilution method of counting nitrifying bacteria; success depends upon the use of a small volume of dilute medium, and a long incubation time.

The *Statistical Tables* of Fisher & Yates refer to more accurate dilution tables than those usually employed; a table of fourfold dilution steps involving the inoculation of two tubes from each dilution is at present being used by the author.

Applications of the Diffusion Technique to the Assay of Certain Antibacterial Compounds. By D. V. CARTER (*Microbiology Division, Standards Department, Boots Pure Drug Co., Ltd., Nottingham*).

Any bacterial compound that will diffuse into an agar gel will give zones of inhibition of growth and will lend itself to a diffusion assay technique. For several compounds a peptone-Lemco medium at pH 8·0 seeded with *Bacillus pumilus* spores gave satisfactory results. Dilutions of the compound were usually prepared in phosphate buffer, but sometimes, as with Hibitane, an acetate buffer was preferred.

The four compounds on which most investigational work has been done are: hexachlorophene (suitable high dilution level, 1 in 10^5; ratio of high to low dilutions, 4:1; very clearly defined zones), bithionol (1 in 10^5; 3:1; fairly good definition), dichlorophane (1 in 10^4; 4:1; fairly good definition), and Hibitane (1 in 10^3; 5:1; good definition but relatively high concentration detracts from usefulness of the test).

The method is particularly useful for hexachlorophene in pharmaceutical preparations when chemical methods are often useless due to interfering substances, e.g. other phenolic compounds. Control preparations of the same constituents as the test sample should be used with the standard solutions. This is necessary because certain materials such as bentonite and kaolin adsorb and retain a proportion of the antibacterial compound even on high dilution, thus giving a low result; certain surface active agents and solubilizing glycols may give high results.

Hexachlorophane in creams, toothpastes, powders and soaps, bithionol in creams and soaps and dichlorophane in powders have all been successfully estimated. With some preparations containing nonionic wetting agents, hexachlorophane gave irregular zones, amoeboid in shape, which rendered the assay invalid. No satisfactory explanation of the phenomenon is known at present.

A Comparison of the Effects of DL-Alanine and Nisin on Spores. By H. B. HAWLEY and G. G. FOWLER (*Biologicals Division, Aplin & Barrett Ltd., Yeovil*).

The effects of DL-alanine and nisin, respectively, on bacterial spores before and after heat treatment have been investigated, using one strain of *Clostridium* and three strains of *Bacillus*. It has been demonstrated that, unlike nisin, DL-alanine has no effect on the apparent D value of spores. The effect of adding DL-alanine to a foodstuff is to stimulate germination of spores prior to thermal processing, so that the reduction in F value becomes apparent to reduction of spore count prior to processing, whereas nisin effects a time reduction by reducing the viability of spores after heating.

The Extraction of Nisin from Meat Products. By G. G. FOWLER and H. B. HAWLEY (*Biologicals Division, Aplin & Barrett Ltd., Yeovil*).

In many foodstuffs, including dairy products, nisin may be assayed directly by dilution assay or by a modification of the cup plate assay procedure following the preparation of an emulsion. Meat protein, however, binds nisin strongly and neither of these procedures is applicable. A procedure is described whereby nisin can be extracted quantitatively from meat and meat products at pH 2·0 using HCl and boiling for 5 min. The supernatant is removed by centrifuging, adjusted to pH 5·5 and assayed by an agar diffusion method.

Afternoon Session. The following papers were read at the Joint Meeting with the Microbiology Group of the Society of Chemical Industry on the subject of

Quantitative Microbiology

Standardization in Quantitative Microbiology. By J. W. LIGHTBOWN (*Department of Biological Standards, National Institute for Medical Research, Mill Hill, London, N.W.7*).

Photometric Methods in Bacteriology. By E. O. POWELL (*Microbiological Research Establishment, Porton, Wiltshire*).

The Use of Bacterial Spore Resistance in Predicting Sterilization Processes. By H. Burton (*National Institute for Research in Dairying, University of Reading*).

The Rapid Bacteriological Testing of Cooked or Cured Meats Using a Tetrazolium Compound. By N. J. Bradshaw and E. J. Dyett (*T. Wall & Sons Ltd., London, W.3*).

ANNUAL GENERAL MEETING AND CONFERENCE

The 31st Annual General Meeting and Conference was held in Wye College on 11, 12 and 13 July, 1961. Members assembled on Monday, 10 July, and the following day was devoted to paper reading sessions in the morning and afternoon with the Annual General Meeting interposed. On Wednesday, 12 July, the morning was devoted to part of a Symposium on 'Bacteria of the Intestine', the afternoon to an excursion to Canterbury or to technical visits to Pfizer Ltd., Sandwich, or the College Farm, and the evening to the Annual Society Dinner. Thursday, 13 July, was occupied by the Symposium.

The following papers were read on 11 July:—

Preliminary Microscopical Observations on Spore Germination in the Genus *Bacillus*. By G. W. Gould (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*).

In a suitable environment spores of *Bacillus* spp. germinate before shedding their outer walls or spore coats. Spores of large species generally lyze their coats and therefore leave behind ill defined structures, but spores of small celled organisms leave well defined coats.

The effects of environmental factors on the way in which spore coats open have been studied. These have included temperature, pH, availability of metals, salt concentration, the presence of coat lyzing enzymes and the effect of preheating spores. Of these, only the pH of the medium markedly affected the type of coat opening. Low pH reduced the extent to which large spores lyzed their coats, and the type of coat opening typical of spores of certain small celled species was changed by low pH.

Some Properties of Aerobic Thermophiles Growing at 65°. By P. D. Walker and J. Wolf (*Department of Agriculture, University of Leeds*).

A collection of 230 aerobic sporeformers capable of growth at 65° and derived from British, American and Dutch sources was submitted to a detailed biochemical study. On the basis of these reactions the organisms fell into three main groups. Variation in the group pattern reactions occurred in groups 1 and 3. Group 2, however, presented an homogeneous pattern and with the exception of some sugar fermentations was characteristically inert. Organisms in groups 1 and 3, however, were much more reactive. Group 1 was characterized by the complete reduction of nitrate to gas, whereas in group 3, reduction did not proceed beyond nitrite. Organisms in the latter group were characterized by their ability to produce considerable acid in milk or tomato-yeast milk and also by a powerful amylase. The extent of variation in groups 1 and 3 was considered in detail.

A New Differential and Diagnostic Medium for *Staphylococcos aureus*. By A. C. BAIRD-PARKER (*Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford*).

Many media have been suggested for isolating and enumerating coagulase positive staphylococci, but they are mostly insufficiently selective or diagnostic. The selective and diagnostic medium described in this paper combines an improved form of the medium of Zebovitz, Evans & Niven with the use of egg yolk as a diagnostic agent.

The composition of the medium (w/v) is: Bacto tryptone, 1·0%; Lab-Lemco, 0·5%; Bacto yeast extract, 0·1%; lithium chloride, 0·5%; agar, 2·0%. The constituents are dissolved and the pH is adjusted to 7·2 before autoclaving at 120° for 15 min. It is dispensed, unfiltered, in 90 ml amounts. To the molten basal medium at about 45° are successively added Seitz filtered solutions of: 20% glycine to a final concentration of 1·2%; 1% potassium tellurite (BDH) to 0·01%; 20% sodium pyruvate to 1·0%; and concentrated egg yolk emulsion (Oxoid) to 5·0% (v/v) (all concentrations being w/v unless otherwise stated).

Each plate should contain 10 ml of freshly poured medium and 0·1 ml of inoculum is spread over the surface. The appearance of colonies of *Staph. aureus* is characteristic. After 24 hr at 37°, black, shiny low-convex colonies are formed, 0·5–1·0 mm in diameter with narrow white entire margins, surrounded by clear zones extending 2–5 mm into the opaque medium. Few egg yolk clearing micrococci grow on this medium; those that do, are distinguished from colonies of *Staph. aureus* by the development of wide inner opaque zones formed in the first 24 hr of incubation.

Ability of the medium to isolate *Staph. aureus* from frozen foods was compared with four other well known media. It was superior to all these media. The medium was also tested against strains of *Staph. aureus* isolated from various foods and human and animal infections. Although selective, the new medium did not suppress *Staph. aureus*, the appearance of which is so characteristic that for routine testing the coagulase test may be omitted.

Geographical Variation in *Pseudomonas solanacearum*. By A. C. HAYWARD (*Commonwealth Mycological Institute, Kew, Surrey*).

A collection of 67 isolates of the soil borne vascular wilt pathogen *Pseudomonas solanacearum*, of diverse host and territory of origin, was examined systematically in the laboratory. On the basis of capacity to oxidize a range of 13 hexoses, hexose alcohols, and disaccharides the isolates were classified into three groups according to extent of oxidative capacity. Type I (37 isolates) was the least active and did not oxidize lactose, cellobiose, or the hexose alcohols mannitol, sorbitol, and dulcitol. Type III (14 isolates) oxidized both disaccharides and the three hexose alcohols, whereas Type II (16 strains) oxidized the disaccharides but not the hexose alcohols.

Type I strains were from hosts in 5 botanical families, and Type III strains from 7, but Type II isolates were exclusively from potato (13 strains) and tomato (1 strain). The limited evidence suggests that Type II strains are of relatively narrow host range. Type I includes the banana pathogen of parts of Central America and the West Indies (Buddenhagen, 1961). Type III may be the *Ps. solanacearum* var. *asiaticum* of Smith (1914). It is probable that in making his original description of the pathogen Smith (1896) worked exclusively with Type I, for in this study all isolates originating in the South Eastern United States possessed the characteristics of this type.

Initial electron microscopical studies on one strain of Type I showed that the cells possessed 4 or 5 polar flagella, some inserted in a subpolar position at right angles to the long axis of the cells, thus suggesting an affinity with, for example, *Rhizobium* (Leifson, 1960) rather than *Pseudomonas*.

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The Effectiveness of White Clover Rhizobia Isolated from Hill Soils. By A. J. Holding and J. King (*Edinburgh School of Agriculture and Hill Farming Research Organisation, Edinburgh*).

Rhizobium strains were isolated from the root nodules of white clover plants growing on 48 hill sites in Scotland. To examine the effectiveness of the rhizobial population of a site, ten strains were added separately to sterile seedlings of white clover (Aberystwyth S. 184) growing on a nitrogen-free mineral salts agar. The *Rhizobium* strains were graded 0–4 according to the growth response of the seedlings, and the sum of the ten grades gave a total score which was considered in relation to certain soil characteristics. The total score for each site was found to be positively correlated with soil base status. The results also indicated that large areas of hill pasture possess a predominantly ineffective indigenous rhizobial population.

Production and Characteristics of Autolysates from Food Yeast. By L. A. Allen (66, *Sutherland Avenue, Petts Wood, Orpington, Kent*).

Three species of yeast, namely *Torulopsis utilis*, *Candida arborea*, and *Zygosaccharomyces lactis*, were selected and each was grown on a molasses medium in a pilot plant designed to produce about 50 lb of yeast solids in a two day run. The yeast was separated from the fermenter liquor, washed and concentrated to a cream of about 14% solids by treatment in a de Laval centrifuge. Pressed yeast, produced by passing the cream through a filter press, was plasmolyzed by mixing it with salt (the amount ranged from 8 to 20% of the yeast solids in different trials) in a stainless steel vessel and the mixture allowed to autolyze at the chosen temperature (usually 50°) for periods varying from 24 to 72 hr. Further enzyme action was stopped by heating the autolysate to 80° for 15 min, then the supernatant liquor obtained after centrifuging was condensed first in a climbing film evaporator and finally in a vacuum pan.

Rates of autolysis of *C. arborea* at various temperatures between 37° and 60° were followed by determining the percentage of yeast solids brought into solution at intervals during incubation of the plasmolyzed yeast. Results showed that a temperature of 50° gave a higher rate of extraction than any other temperature except that, if a very short period of autolysis (18 hr or less) were to be employed, a temperature of 55° might give a somewhat greater breakdown.

The contents of amino acids, purines, fat, carbohydrate and inorganic constituents were also determined.

Extracts of acceptable flavour were produced from both *C. arborea* and *Z. lactis* but not from *T. utilis*. One factor which appeared to affect the flavour very considerably was the chemical interaction between the breakdown products of the carbohydrate and the protein. This effect was enhanced by raising the temperature of the extract as the process neared completion in the vacuum pan.

The work described formed part of the investigation on food yeast carried out in the Molasses Utilization Department of the Tate & Lyle Research Laboratory.

The Production of Hydrogen Sulphide by Washed Suspensions of *Escherichia coli*. By D. H. Shrimpton (*Low Temperature Research Station, Cambridge*).

The production of hydrogen sulphide in the bowel of chickens has long been assumed (Pennington & Sherwood, 1922). More recently Barnes & Shrimpton (1957) demonstrated its production *in situ* in newly killed birds, finding the greatest concentrations of bacteria in the caecum and large intestine where coli-aerogenes bacteria constituted one of the major groups; Beattie & Shrimpton (1958) observed the production of H_2S from the caeca of live anaesthetized birds. Later work (Shrimpton, 1958) indicated that the amounts produced were influenced by the amount of carbohydrate in the diet and by the pH of the system, the highest rate of production being at pH 5·6 and the lowest at 7·8. A systematic study of the groups of bacteria responsible for this production and its mechanism was started and a brief account is given of work with isolates of *Escherichia coli*.

Washed suspensions of about 10^9 cells/ml were incubated in an atmosphere of nitrogen at 37° for 2 hr in Thunberg tubes which were shaken once a second and inclined at 25° to the horizontal. Hydrogen sulphide was collected in 0·1 N Na_2CO_3 in the bulb of the Thunberg tube and estimated colorimetrically with dimethyl-*p*-phenylenediamine (Lauth's reaction) after the method of Almy (1925). A basal medium of tryptone-meat extract broth was supplemented with a variety of substrates containing sulphur and buffered at pH values between 4·5 and 9·0.

Thirty-seven isolates were studied and it was concluded that H_2S was produced by the L-cysteine desulphydrase system of Desnuelle & Fromageot (1939). When the isolates were classified according to their rate of production of H_2S at pH 6·0 and pH 8·0 there were 4 groups: (1) similar at each pH; (2) that at pH 6·0 double that at pH 8·0; (3) no activity at pH 8·0; and (4) no activity at either pH.

The inhibitory effect of glucose reported by Binkley (1943) was observed when the substrate was L-cysteine, but when this was replaced with L-cystine the effect of the glucose was stimulatory, presumably because of its action as a hydrogen donor (Desnuelle, 1939).

These observations made *in vitro* are compatible with those observed *in vivo* and it is proposed that at least some of the H_2S produced in the caecum results from the action of a L-cysteine desulphydrase system.

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Observations on the Secular Succession of Types of *Escherichia coli* and Related Organisms in the Faecal Flora of an Adult Human Subject.

By A. H. Emslie-Smith (*Department of Bacteriology, King's College, Newcastle-upon-Tyne*).

Over a period of 30 months ten quantitatively representative strains, assignable to the Enterobacteriaceae, were isolated from each of 76 faecal specimens from a healthy adult. These strains were typed according to metabolic activities, colicine production and sensitivity to colicines and selected phages. Some 40 types and sub-types were recognized. Only 7% of the strains, comprising twenty types, were found to be other than typical *Escherichia coli*; 85% of all strains could be assigned to one or other of three types and 75% of the specimens yielded 'pure cultures' (all 10 strains alike) of these types which have been designated *dynastic*. All dynastic strains were colicinogenic, but only strains belonging to the first dynasty were sensitive to the colicines used. Fimbriation does not appear to be a character necessary in a dynastic type.

The dynastic types appeared in succession and were not found together. The first dynasty was present in 'pure culture' during the first 2 months of the investigation, but was not recovered after the spontaneous appearance of strains producing a colicine active against it. The second dynasty was found within the next month but took some 9 months to establish as the exclusively predominating coli-aerogenes type and during this period the vast majority of the nondynastic (transient) types (15% of all strains) were isolated in small numbers from single faecal specimens. Subsequently the second dynasty was exclusively predominant for 8 months and only disappeared 3 weeks after the oral administration of a mixture of 3 phages active against a type strain and variants of it resistant to one or more of these phages. The disappearance of the second dynasty was followed immediately by the appearance of strains belonging to the third dynasty and these have remained exclusively predominant except during and just after an acute intestinal upset and, recently, following a course of immunization with a formolized autogenous vaccine of a representative third dynasty strain.

The Incidence of Lecithinase Positive *Clostridium welchii* in the Pig, and the Effects Thereon of Antibiotic Food Supplements.

By O. ANDRLA and C. A. E. BRIGGS (*Veterinary and Agricultural Research Department, Pfizer Ltd., Sandwich, Kent*).

It is well known that the growth of young animals and humans is improved by the addition of antibiotics to their diet. In the chick this effect is generally attributed to the bacteriostatic action of antibiotics on lecithinase positive *Clostridium welchii*. It has been found in the pig that these organisms are not present between 15 and 30 weeks of age, though there is ample evidence of numerous workers that statistically significant growth stimulation by antibiotics at this age does occur. It has been shown in an experiment with 18 pigs (which had never received antibiotics and which came from stock not fed antibiotics) that, prior to the disappearance of lecithinase positive *Cl. welchii* at 15 weeks of age, lecithinase activity decreased progressively and finally ceased. Subsequent reappearance of the organisms at 30 weeks of age was followed by gradually increasing lecithinase activity. The indication that natural antibiosis may be functional in the gut of the pig of 15–30 weeks of age was supported by the reproduction of this phenomenon on feeding antibiotic supplements to older pigs. It is of importance that *Cl. welchii* Type A, which is often implicated as a cause of disease in animals and man, may be difficult of detection by reason of its temporary atoxigenicity in animals and humans receiving antibiotic therapy or feed supplements. The effect of antibiotic feed supplement on α-toxin production of *Cl. welchii* in older animals may be of some prophylactic value.

The Gastrointestinal Flora of the Dog.

By ELIZABETH M. HARPER and W. L. WEIPERS (*Department of Bacteriology and Department of Veterinary Surgery, The University, Glasgow*).

Clostridium welchii type A is a commensal of the gastrointestinal tract of the normal dog, being readily isolated from the stomach contents, the duodenum and ileum. Associated with this organism in these sites is a varying flora which may contain, for example, *Staphylococcus aureus*, haemolytic and nonhaemolytic streptococci, *Streptococcus faecalis*, coli-aerogenes bacteria, *Proteus* spp., *Pseudomonas* spp., *Cl. bifermentans*, and *Cl. sporogenes*. Surgical obstructions were made in the small intestine of dogs and material taken from this 48 hr after operation was examined bacteriologically. On culture there was an overwhelming growth of *Cl. welchii* type A. The other bacteria isolated included *Sphaerophorus* spp., *Cl. sporogenes*, other *Clostridium* spp., diphtheroids, *Strep. faecalis*, other streptococci, coli-aerogenes bacteria, and *Proteus* spp. The dogs died 4 days after the operation with symptoms of toxaemia. Administration of *Cl. welchii* antitoxin (8,000–20,000 units/ml) at the time of operation protected the dogs against death. The antitoxin had no direct effect on either the numbers of *Cl. welchii* found or on the other bacterial species present in the obstruction, so it is probably correct to assume that the beneficial action of the antitoxin was due to its neutralizing effect on the *Cl. welchii* toxins. Penicillin also protected against death. One dog received penicillin injected directly into the obstruction at the time of operation, as well as parenterally, and was still alive 21 days later. *Cl. welchii* was inhibited by the antibiotic: samples taken 72 hr after operation showed no growth of the bacteria in the presence of penicillinase.

The Occurrence of Luminous Bacteria in the Intestinal Tract of Marine Fish. By R. Spencer (*Humber Laboratory, Department of Scientific and Industrial Research, Hull*).

Luminous bacteria were shown to be present in the intestinal tract of 152 out of 199 individuals, representing 36 species and 17 families of fish, caught in areas from south east Iceland to the west English Channel at various times of the year. Two species of luminous bacteria, *Photobacterium phosphoreum* and *Ph. fischeri*, formed all but one of 733 isolates. There were strong indications that the proportions of these two species of bacteria varied from group to group of fish examined, although not from fishing area to fishing area. The highest proportion (94%) of *Ph. phosphoreum* was found in herrings (*Clupeidae*) and the highest proportion (43%) of *Ph. fischeri* in flat fish (*Pleuronectidae*). These differences may be related to feeding habits.

Some Functions and Malfunctions of Micro-Organisms in the Human Gut. By Margaret A. Long (*Metabolic Research Unit, Little Bromwich Hospital, Birmingham*).

The small intestine in the healthy human subject has been shown to be free from a resident flora. In patients with malabsorptive disorders, food materials are incompletely absorbed and this, often combined with low gastric acidity and depressed motility, provides an ideal environment for bacterial proliferation. Folic acid deficiencies frequently occur and a variable amount of faecal fat of nondietary origin is excreted. Organisms capable of *in vitro* degradation of folic acid have been isolated from the duodenal juices of such cases.

The marked reduction in faecal fat levels after antibacterial therapy and the qualitative and quantitative examination of microbical fat inclusions lead to the conclusion that in certain cases micro-organisms may make a substantial contribution to the abnormally high content of faecal fat.

The following papers were read on 12 July in the Symposium on

Bacteria of the Intestine

The Bacterial Flora of the Faeces of Animals with Particular Reference to its Development in the Young. By H. Williams Smith (*The Animal Health Trust, Farm Livestock Research Centre, Essex*). (See *J. appl. Bact.* **24**, 235).

Microecological Rules in the Composition of the Intestinal Flora of Man. By H. Haenel (*Institut fur Ernährung, Potsdam-Rehbrücke*). (See *J. appl. Bact.* **24**, 242).

Studies on the Bacterial Flora in the Alimentary Tract of Pigs: 1. Enterobacteriaceae and Other Gram-Negative Bacteria. By Anne B. Dickinson and G. Mocquot (*Station Centrale de Recherches Laitières et de Technologie des Produits Animaux, Jouy-en-Josas, France*). (See *J. appl. Bact.* **24**, 252).

Studies on the Bacterial Flora in the Alimentary Tract of Pigs. 2. Streptococci: Selective Enumeration and Differentiation of the Dominant Groups. By P. Raibaud, M. Caulet, J. V. Galpin and G. Mocquot (*Station Centrale de Recherches Laitières et de Technologie des Produits Animaux, Jouy-en-Josas, France*). (See *J. appl. Bact.* **24**, 285).

The following papers were read on 13 July:—

Germ Free Animals and Their Use in Elucidating the Action of the Gut Flora on the Host. By M. Lev (*National Institute for Research in Dairying, University of Reading*). (See *J. appl. Bact.* **24**, 307).

Host Specificity and Enteropathogenicity of *Escherichia coli*. By Joan Taylor (*Salmonella Reference Laboratory, Central Public Health Laboratory, London, N.W.*9). (See *J. appl. Bact.* **24**, 316).

Studies on the Growth, Sporulation and Carriage of *Clostridium welchii* with Special Reference to Food Poisoning Strains. By J. G. Collee, Jill A. Knowlden and Betty C. Hobbs (*Central Public Health Laboratory, Colindale*). (See *J. appl. Bact.* **24**, 326).

The Public Health Significance of Salmonella Carriers in Livestock and Birds. By Betty C. Hobbs (*Central Public Health Laboratory, Colindale*). (See *J. appl. Bact.* **24**, 340).

The Significance of Organisms of Supposed Faecal Origin in Foods and Water. By R. Buttiaux and D. A. A. Mossel (*Institut Pasteur de Lille*). (See *J. appl. Bact.* **24**, 353).

This paper was followed by two relevant contributions:—

The Safety and Cleanliness of Waters and Foods. By J. H. McCoy (*Public Health Laboratory, Kingston-upon-Hull*). (See *J. appl. Bact.* **24**, 365).

Some Observations on Coli-Aerogenes Bacteria and Streptococci in Water. By N. P. Burman (*Metropolitan Water Board, London, E.C.*1). (See *J. appl. Bact.* **24**, 368).

AUTUMN MEETING, 1961

The Autumn Meeting of the Society was held on Tuesday, 24 October, 1961, at Queen Elizabeth College, London, W.8, and comprised an Address on The Philosophy of Disinfectant and Antiseptic Evaluation given by the President, Mr. G. Sykes, followed by demonstrations.

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